University of Alberta

PROMOTING PERIPHERAL NERVE REGENERATION: FUNCTIONAL ELECTRICAL STIMULATION AND PHARMACOLOGICAL APPROACHES

By

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Department of Pharmacology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Promoting Peripheral Nerve Regeneration: Functional Electrical Stimulation and Pharmacological Approaches** submitted by **Abdulhakeem A. Al-Majed** in partial fulfilment of the requirements for the degree of **Doctor of Philosophy**.

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ABSTRACT

Functional recovery after peripheral nerve transection is often poor despite the capacity of the axons to regenerate and advances in microsurgical technique. Misdirection of regenerating axons to inappropriate targets is a major contributing factor for this failure. In this study, we use the adult rat femoral nerve model of transection and surgical suture to evaluate (1) the effect of nerve transection on the speed and accuracy of motor axonal regeneration, (2) the efficacy of 20 Hz continuous electrical stimulation in accelerating axonal regeneration and promoting specificity, and (3) investigate the mechanism (s) of action of stimulation: (a) site of action, and (b) effects of stimulation on gene expression of brain-derived neurotrophic factor (BDNF), it's high affinity receptor, trkB, medium molecular weight neurofilament protein (NFM), Tα1-tubulin and GAP-43 in regenerating motoneurons. Axonal regeneration was evaluated by a backlabeling technique to enumerate motoneurons which regenerate using retrograde neurotracers, fluorogold and fluororuby. Levels of gene expression were semiquantitated using *in situ* hybridization technique.

Enumeration of backlabeled regenerating motoneurons, revealed that: (1) there is a very protracted period of axonal outgrowth (staggered regeneration). This process is associated with preferential motor reinnervation (PMR), (2) 1 hr to 2 weeks of stimulation of the parent axons proximal to the transection and repair site applied immediately after nerve suture promotes the speed and accuracy of motor axonal regeneration, and (3): (a) the positive effect of 1 hr stimulation is mediated

via the cell body, and (b) stimulation leads to rapid upregulation of BDNF and trkB mRNA in regenerating motoneurons. This is followed by rapid and dramatic downregulation of NFM mRNA, concurrent with a large upregulation of Tα1-tubulin and GAP-43 mRNA.

In conclusion, the effectiveness of only a brief 1 hr period of electrical stimulation in promoting speed and accuracy of axonal regeneration after femoral nerve cut and suture suggests a new therapeutic approach to accelerate peripheral nerve regeneration after injury and, in turn, improve functional recovery. Mechanistically, our results support the hypothesis that: (1) acceleration of BDNF upregulation by stimulation of axotomized motoneurons, and (2) the reduction in NFM/tubulin expression ratio by stimulation of regenerating motoneurons, play key roles in accelerating of axonal outgrowth.

DEDICATION

This thesis is dedicated to my mother whom this entire adventure would never have been possible

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2.

ABBREVIATIONS

BDNF:	brain-derived neurotrophic factor
CNS:	central nervous system
GAP-43:	growth-associated protein-43
FG:	fluorogold
FR:	fluororuby
ISH:	*in situ* hybridization
NFM:	medium-molecular-weight neurofilament protein
NT:	neurotrophin
NGF:	nerve growth factor
NT-3:	neurotrophin-3
NT-4/5:	neurotrophin-4/5
NT-6:	neurotrophin-6
PMR:	preferential motor reinnervation
PNS:	peripheral nervous system
RAGs:	regeneration associated-genes
SCb:	slow component b
Tα1-tubulin:	total α1-tubulin
trkB:	tropomyosin receptor kinase B
TTX:	tetrodotoxin

CHAPTER 1

1.0 GENERAL INTRODUCTION:

PERIPHERAL NERVE INJURY

1.1 INTRODUCTION

Adult mammalian axonal regeneration is generally successful in the peripheral nervous system (PNS) but is dismally poor in the central nervous system (CNS) (for review, see Fu and Gordon, 1997; Yin et al., 1998). A comparison of myelin from the CNS and the PNS has revealed that CNS white matter is selectively inhibitory for axonal outgrowth (Schwab and Thoenen, 1985). Several components of CNS white matter, NI35, NI250 (Nogo) and myelin associated glycoprotein (MAG), that have inhibitory activity for axon extension have been described (Caroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhay et al., 1994; Spillmann et al., 1998; Chen et al., 2000; Grandpre et al., 2000). However, many classes of CNS axons can extend for long distances in peripheral nerve grafts (Benfy and Aguayo, 1982). The Schwann cell environment supports regeneration of both central and peripheral neurons and their absence leads to failure of even PNS regeneration (for review, see Fu & Gordon, 1997). Despite the permissive environment of Schwann cells, functional recovery after peripheral nerve injury is variable, often very limited and rarely returns to the preinjury levels particularly if the injury requires regeneration over long distances and, thus, months and even years of regeneration (Kline & Hudson, 1995; for review, see Sunderland, 1978). These periods are much longer than predicted from reported regeneration rates of 1-3 mm/d, and the latent period of 3-7 d for axons to regenerate across the surgical site and to form functional connections with denervated targets (Gutmann et al., 1942; Sunderland, 1947, 1978; Kline & Hudson, 1995).

There are many possible reasons for poor outcome, some of which are related to adventitious factors such as the nature and site of the injury, the presence of associated injuries, poor blood supply, delay between injury and repair which leads to deterioration of the growth potential of axotomized neurons and the permissive environment of the distal nerve stump and end-organ (Fu and Gordon, 1995a,b; for review, see Brecknell and Fawcett, 1996; Fu & Gordon, 1997), and poor surgical alignment of stumps. Outcome is also limited by more general factors of the biology of the nervous system, such as the limited rate and accuracy of nerve regeneration. Despite early diagnosis and modern microsurgical techniques which give accurate nerve stump coaptation, functional recovery is rarely complete. Optimal recovery will occur when maximal numbers of axons of an appropriate size and type reach the correct target organ in the shortest time after injury, thereby reducing the deterioration of the permissive environment of the distal nerve stump and end-organ associated with denervation (Fu and Gordon, 1995a,b; Whitworth et al., 1996). For this reason, achieving nerve regeneration at a faster rate and in higher volumes is well recognized to be a key in optimizing functional recovery.

1.2 DISTAL NERVE STUMP RESPONSE TO AXOTOMY

1.2.1 Wallerian degeneration

Axotomy of a peripheral nerve leads to interruption of axonal integrity with ensuing degeneration of nerve fibres distal to the site of transection (distal stump), a process named Wallerian degeneration (Waller, 1850). During Wallerian degeneration a microenviroment is created in the distal nerve stump that allows successful regrowth of nerve fibers from the proximal nerve segment. Wallerian degeneration in the distal stump begins with prompt degradation of axoplasm and axolemma induced by the activation of axonal proteases and calcium influx (Schlaepfer and Bunge, 1973). Within 2 days after nerve axotomy, Schwann cells distal to the injury site sequester small whorls of myelin debris and fragment their own myelin sheaths into ovoids (Liu et al., 1995; Stoll et al., 1989). Schwann cells phagocytose myelin debris to some extent and form lipid droplets before macrophages enter degenerating nerves (for review, see Fu and Gordon, 1997; Stoll & Müller, 1999). After the initial extrusion of myelin sheaths, Schwann cells divide to a maximum at day 3 (Stoll et al., 1989; Carrol et al., 1997) and line up within the basal lamina tubes to form bands of Büngner, that later guide regenerating nerve fibres into the endoneurial tubes to reinnervate denervated targets (for review, see Fu and Gordon, 1997; Stoll & Müller, 1999). Beginning on day 2 and reaching a maximum between days 4 to 7, hematogenous macrophages enter the distal stump and migrate to the ovoids. Within 2 weeks macrophages completely clear myelin debris (Stoll et al., 1989; Liu et al., 1995; Brück, 1997; for review, see Fu and Gordon, 1997; Stoll & Müller, 1999).

During Wallerian degeneration a machinery of molecular changes take place in the perikarya as well as in the distal degenerating stump of the injured motor and sensory neurons. These changes will be described in brief in an accompanying section of this chapter.

1.2.2 Molecular response in the distal nerve stump

Transection of a peripheral nerve leads to a dramatic change in the molecular composition of the distal nerve segments (DeLeón et al., 1991; Gillen et al., 1995; Gillen et al., 1997). Thereby, a microenviroment develops that supports axonal regeneration in the PNS and, moreover, allows elongation of usually non-regenerating transected CNS fibre tracts into grafted PNS nerve segments (Aguayo et al., 1982). Molecular changes include upregulation of the following: i) neurotrophins such as nerve growth factor (NGF) (Funakoshi et al., 1993) and brain derived neurotrophic factor (BDNF) (Meyer et al., 1992), and their low affinity (common) neurotrophin receptor p75 (Heumann et al., 1987a,b; Taniuchi et al., 1988; Funakoshi et al., 1993; for review, see Fu and Gordon, 1997); ii) glial-cell-line-derived neurotrophic factor (GDNF) (Trupp et al., 1995); iii) neural cell adhesion molecules such as the cell adhesion molecule, L1, and neural cell adhesion molecule (N-CAM) (Martini and Schachner, 1988; Bosch et al., 1989; Johnson et al., 1990;) and iv) cytokines such as the interleukins, IL-1, IL-6 and IL-10 (Bolin et al., 1995; Bourde et al., 1996; Reichert et al., 1996; Gillen et al., 1998). These changes occur concomitant to the downregulation of myelin associated proteins such as myelin basic protein (MBP), MAG, protein zero (P_0) (for review, see LeBlanc and Poduslo, 1990; Stoll & Müller, 1999).

1.3 RESPONSE OF NEURON TO AXOTOMY

1.3.1. Changes in gene expression

The early response of the neuronal perikaryon to axotomy includes chromatolysis and a major shift in gene expression, such as the prominent upregulation of growth associated genes, most conspicuously GAP-43 (for review, see Skene, 1989; Benowitz and Routtenberg, 1997; Fu and Gordon, 1997), immediate early genes such as c-jun (for review, see Herdegen et al., 1997), and cytoskeletal proteins such as tubulin and actin (for review, see Bisby and Tetzlaff, 1992). These changes occur concomitant with the downregulation of neurofilament (Bisby and Tetzlaff, 1992) and in motoneurons neurotransmitter enzymes (Fernandes et al., 1998; Kou et al., 1995).

It has been proposed that axonal elongation is achieved by an elongation of the axonal cytoskeleton which is synthesized and assembled at the cell body and which then moves down the axon either as a continuous network or as individual polymers (Lasek, 1986; Lasek and Katz, 1987). There is a close correlation between the rate of slow component b transport of tubulin (SCb) and the rate of axonal regeneration (1-3 mm/d) (McQuarrie, 1983; Wujek and Lasek, 1983). This correlation, together with the evidence of upregulation of tubulin mRNA and downregulation of neurofilament proteins after axotomy (Tetzlaff et al., 1988), strongly supports the view that regenerating axons recapitulate development, transporting increased supplies of tubulin to the growth cones for axonal growth (Hoffman and Cleveland, 1988). Downregulation of neurofilament proteins has been suggested to increase the fluidity of the axoplasm and thereby facilitate axonal transport of tubulin and more rapid axonal regeneration (Tetzlaff et al., 1996). However, the precise relationships between transport of tubulin, reduced transport of neurofilaments, and regeneration are yet to be established (for review, see Bisby, 1995; Fu and Gordon, 1997).

In addition, axotomy of motoneurons lead to loss of retrogradely transported trophic factors such as BDNF from muscle target (Kristenson and Olson, 1975). Axotomized motoneurons respond to axotomy by increasing the expression of low-affinity neurotrophin receptor, p75 (Ernfors et al., 1989), as well as trkB, the signal transducing receptor for BDNF and neurotrophin NT-4/5 (Meyer et al., 1992; Funakoshi et al., 1993; Piehl et al. 1994; Kobayashi et al., 1996; for review, see Fu & Gordon, 1997). Furthermore, axotomized motoneurons upregulate GDNF and its signal transduction component, the tyrosine kinase RET (Naveillan et al., 1997). Additional changes include downregulation of glutamate receptor expression which is "probably associated with the loss of synapses impinging upon motoneurons after axotomy, although the causal relationship between the two phenomena remains to be explored" (Popratiloff et al., 1996) and a transient increase in calcitonin gene related peptide (CGRP) immunoreactivity (Arvidsson et al., 1990).

In axotomized sensory neurons, the neuropeptides, substance P, CGRP, and somatostatin, which are normally expressed in the transmitting neurons, are downregulated whereas vasoactive intestinal peptide, galanin, neuropeptide Y, and cholecystokinin are upregulated (Verge et al., 1996; for review, see Zigmond et al., 1996). Moreover, axotomy induces a marked upregulation of nitric oxide synthase in primary sensory neurons (Verge et al., 1992; Zhang et al., 1993).

1.3.2 Formation of growth cones

After a nerve injury where the axons are interrupted, the proximal axonal stump undergoes traumatic Wallerian degeneration to the first uninjured node of Ranvier (Cajal, 1928). Nerve fibres undergo outgrowth from the proximal stump, known as the growth cone, consisting of multiple axonal sprouts (filopodia) surrounded by Schwann cells. Since first described by Cajal (1928), growth cones have been studied extensively as the specialized structures responsible for growth, pathfinding and recognition of targets (Landis, 1983). Electron microscope studies have defined the characteristic features of growth cones including accumulations of membranous organelles and organized cytoskeletal components (Tennyson, 1970; Yamada et al., 1971; Bunge, 1973; Bridgman and Dailey, 1989) which are primarily tubulin and actin. It is generally believed that the extension of axons is carried out by the supply of components to the growth cone plasma membrane (Feldman et al., 1981; Griffin et al., 1981; Pfenninger and Maylie-Pfenninger, 1981; Craig et al., 1995). Organized cytoskeletal structures in association with intracellular signal transduction determine the shape and motility of growth cones (Igarashi and Komiya, 1991; Sobue, 1993; Change et al., 1995). Intracellular calcium regulates the motility of growth cones (Goldberg, 1988; Gomez et al., 1995). Growth cones use proteases in order to extend through the tissue matrix (Seeds et al., 1992). They extend through their basal lamina tubes in the proximal segment, traverse the narrow gap of connective tissue between the proximal and distal stumps, and finally enter the space between the basal lamina and the Schwann cell plasma membrane or myelin sheath in the distal stump (for review, see Ide and Kato, 1990; Ide, 1996). The axonal sprouts extend by attaching themselves to the inner surface of the basal lamina or on the Schwann cell plasma membrane (for review, see Ide, 1996). Laminin, a potent axon outgrowth-promoting factor and the major component of basal lamina, serves as contact guidance to the filopodia. The axonal sprouts that do not make synaptic contact with the target undergo degeneration (Cajal, 1928; for review, see Ide, 1996; Fu and Gordon, 1997; Dagum, 1998).

1.4 AXONAL REGENERATION

1.4.1 Injury and misdirection of regenerating axons

The nature of nerve injuries determines prognosis, treatment and functional outcome after a peripheral nerve injury. For axonal regeneration to fully restore functional recovery after nerve injuries, axons must be guided back to their original targets by Schwann cells in the endoneurial tubes which were formerly occupied by intact axons and their myelin sheaths. In crush injuries, where there is axonal disruption but the continuity of the endoneurial tube remains intact, all axons regenerate and make specific connections. Crush injuries are associated with successful regeneration and complete functional recovery. Conversely, in transection injuries, where the nerve is completely severed and requires surgical repair, and regenerating axons must cross a gap between the proximal and distal nerve stumps, many axons are misdirected to reinnervate inappropriate targets which often leads to poor functional recovery (for review, see Sunderland, 1978; Fu and Gordon, 1997; Dagum, 1998). Despite advances in microsurgical techniques which aim to align the cut nerve stumps in order to facilitate regeneration of axons back to their targets, the axons are frequently misdirected to inappropriate targets, rather than their original targets. Random matching of regenerating proximal axons with Schwann tubes in the distal stump is thought to be an important factor contributing to this misdirection (for review, see Sunderland, 1978). Regenerating motor axons could enter distal Schwann tubes which previously contained sensory axons, and be led inappropriately to innervate sensory end organs. Similarly, regenerating sensory axons could enter old motor Schwann tubes and be directed to denervated motor end plates. Not only would these misdirected axons fail to establish functional contacts, they would also exclude appropriate axons from the pathways they originally occupy. However, Brushart (1988,1993) demonstrated that, after transection and surgical repair of a mixed sensory-motor nerve such as femoral nerve, motoneurons display preferential reinnervation of the muscle branch when given equal access to muscle and cutaneous pathways in both juvenile rats at 3 weeks and adult rats at late stages of regeneration (8 and 12 weeks after nerve transection). This process is referred to as preferential motor reinnervation (PMR) (Brushart, 1988). In addition, recent results have shown that PMR takes place in the nonhuman primate (Madison et al., 1999).

PMR occurs even in the absence of a target, suggesting that a specific interaction occurs between regenerating motor axons and the Schwann cell tubes that lead the axons into the motor branch (Brushart, 1990,1993). This suggests that certain permissive factors for motor axons must be present in the motor branch which are not present in the sensory branch. A pathway marker and guidance molecule has been recently identified in mammals (Peinado, 1987) which is associated with the generation of PMR (Martini et al., 1994). This is L2/HNK-1, a carbohydrate epitope on cell adhesion molecules (Kruse et al., 1984). The L2/carbohydrate epitope was originally described as a cell surface component of human natural killer cells and is common in a large family of recognition molecules (for review, see Martini, 1994). The L2/carbohydrate is selectively expressed on the Schwann cells and Schwann cell basement membranes of motor axons, but is rarely found on sensory axons (Martini et al., 1994). In the early stages of axonal regeneration the L2/carbohydrate is expressed by Schwann cells that were previously associated with motor axons. However, after 21 days there is little or no L2 expression by the Schwann cells if they remain denervated. Further experiments demonstrated that, during regeneration of transected and sutured the rat femoral nerve, previously motor axon associated Schwann cells dramatically re-expressed L2 when reinnervated by motor axons, but not when reinnervated by sensory axons. In contrast, previously sensory-associated Schwann cells did not express L2 when contacted by a motor or a sensory axon (Martini et al., 1994). This interaction between the motor axons and motor axon-associated Schwann cell along with L2/HNK-1 expression during critical stages of reinnervation may underlie the preferential regeneration of motor axons in the appropriate muscle pathways.

Recently, anatomical evidence for specificity was also provided during regeneration of sensory afferent projections to muscle (Madison et al., 1996). The accuracy of sensory afferent regeneration was highly correlated with the accuracy of motor regeneration suggesting that two distinct neuronal populations that project to muscle respond in parallel to specific guidance factors during regeneration process. The accuracy of pathway finding of sensory fibres to skin has not been addressed further and the signal molecules involved remain unknown.

1.4.2 Surgical repair

The goal of surgical repair of injured peripheral nerves is to restore innervation and function. This means providing an environment that will maximize axonal regeneration with optimal fascicular orientation and thereby promote target reinnervation. Nerve repair is performed using standard microsurgical techniques, with epineurial repair being the gold standard with which other types of repair are compared. The proximal and distal stumps are carefully aligned under the microscope, then an epineurial repair is performed by the placement of microsutures (9-0, 10-0) through the external epineurium. Tubes such as silicone tubes (Brushart, 1987, 1988; Lundborg et al., 1991) are used to provide a conduit through which the proximal nerve end grows to meet the distal end (i.e mechanical guidance). The theoretical advantages include prevention of ingrowth of scar tissue and outgrowth of axonal sprouts to non-nerve sites and provision for prolonged delivery of exogenous neurotrophic factors when they become better defined and available (for review, Dagum, 1998).

Tissue adhesives such as fibrin glue have been used for nerve repair. The long-term results have generally been inferior or equal to those of conventional nerve repair. Laser nerve repair which causes a small amount of the approximated nerve ends to make them adhere to each other, much like a spot weld. Again, in most studies these techniques have proved inferior to the conventional nerve repair (for review, see Terris and Fee, 1993; Dagum, 1998).

1.4.3 Methods to accelerate nerve regeneration and promote PMR

There are presently no established clinical methods to promote axonal regeneration and complete recovery after peripheral nerve transection injuries. The effectiveness of several experimental approaches have been evaluated almost exclusively for crush injuries and not others. These approaches include using a conditioning lesion, direct current stimulation, alternating electrical stimulation, the L-type Ca^{2+} channel blocker nimodipine, FK506 and neurotrophins. Evaluation of these methods to promote peripheral nerve regeneration has been limited but in some cases, the approaches are promising. These are discussed in turn in the following sections. Approaches such as the use of sinusoidal 50 Hz magnetic field stimulation (Rusovan and Kanje, 1992) which have been evaluated for improving rate of peripheral nerve regeneration have limited positive effects in rats after sciatic nerve crush injury. In addition, agents such as melanocortins, adrenocorticotrophic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), testosterone, gangliosides in diabetic animals, insulin, insulin like growth factor (Bijlsma et al., 1984; Strand and Smith, 1986; DeKoning and Gispen, 1987; Ekström et al., 1989; Ekström and Tomlinson, 1990; Sporel-Özaka et al., 1990; Jones, 1993; Roth et al.,

1995) and others have been experimentally examined. These agents have positive but small effect on the rate of regeneration in the adult rat after crush injuries but their effectiveness has not been evaluated after transection injuries. In addition, the potential toxicities of these agents have limited their use in the therapy of peripheral nerve injury. For example, the use of gangliosides which are the most widely lauded of these agents to promote peripheral nerve regeneration after injury has been discouraged because of the development of Guillain-Barré syndrome (Landi et al., 1993)

1.4.3.1 Conditioning lesion

Several studies have shown that axonal outgrowth is significantly enhanced after a "test" lesion (crush injury) if a peripheral nerve has been subjected to a suitably timed and spatially located "conditioning" lesion (crush injury) (for review, see Fu and Gordon, 1997). The conditioning lesion is given prior to the test lesion and in different studies, the location of the conditioning lesion relative to the test lesion is either proximal, distal or at the same point as the test lesion (McQuarrie, 1973,1985; Bisby and Pollock, 1983; Sjöberg and Kanje1990). This conditioning lesion effect has been observed in both mammalian sensory and motor axons (McQuarrie, 1973,1985; Forman and McQuarrie, 1980; Bisby and Pollock, 1983; Bisby, 1985; Sjöberg and Kanje1990), as well as in frog sciatic nerve (Carlsen, 1983; Edström and Kanje, 1988) and goldfish optic nerve (Lanners and Grafstein, 1980; Edwards et al., 1981; McQuarrie and Grafstein, 1982). The optimal conditioning interval irrespective of the location of the conditioning lesion may be as long as 2 weeks (Forman and McQuarrie, 1980; Edwards et al., 1981). However, shorter intervals of 2 days (Forman and McQuarrie, 1980) or even 14 h (Arntz et al., 1989) between conditioning and test lesions result in accelerated outgrowth.

The mechanism by which a prior conditioning lesion is able to accelerate axonal regeneration after crush injuries is not yet known. There are two main hypotheses. One is that outgrowth is enhanced in conditioned nerves since they are already in a state of regeneration when the second test lesion is made. Thus their axotomized cell bodies have made the necessary adjustments (Grafstein and McQuarrie, 1978) and the axons contain the materials required for regeneration. The cell body adjustment includes upregulation of growth-associated proteins and their transport into the axon prior to the outgrowth of nerve sprouts (McQuarrie and Grafstein, 1982; McQuarrie and Jacob, 1991; Tetzlaff et al., 1996). The other hypothesis is that the local environment surrounding the growth cone controls the rate of axonal elongation (Brown and Hopkins, 1981; Sjöberg and Kanje, 1990). Thus, changes in non-neuronal cells, including the proliferation of Schwann cells (Thomas, 1970; for review, see Fu and Gordon, 1997) and production of trophic factors (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993; for review, see Johnson et al., 1988; Fu and Gordon, 1997), are induced by the conditioning lesions when made proximal to or at same site as the test lesion and could account for the increased outgrowth. These conditioning lesions cause Wallerian degeneration in the distal stump prior to the test lesion and thereby provide vacant channels for axonal growth. In other words when the test lesion is made the axons are thought to grow more rapidly along pathways vacated by the pre-degenerated axons (Brown and Hopkins, 1981; Sjöberg and Kanje, 1990).

The first hypothesis (cell body reaction) is supported by an early observation that nerve regeneration cannot be sustained in frogs kept at 15 C°, a temperature at which there was no cell body reaction, unless the cell body was activated by a prior conditioning lesion (Carlsen, 1983). In addition, a wide spatial separation of the conditioning lesion distal to the test lesion which activates the cell body reaction without priming the distal nerve stump, does not prevent the conditioning lesion effect (Forman et al., 1980; McQuarrie and Grafstein, 1982; Oblinger and Lasek; 1984; McQuarrie and Jacob, 1991). Hence the conditioning lesion effect is one exerted on the neuron itself and cannot be explained simply by axonal regeneration through a pre-degenerated local environment (McQuarrie, 1985). The influence of the cell body response to the conditioning lesion is emphasized by experiments where the location of conditioning lesion distal to the proximal test lesion meant that the conditioned axons grew through freshly lesioned nerve, and pre-degeneration was not involved (McQuarrie and Grafstein, 1973; Bisby and Pollock, 1983).

The effects of the conditioning lesion on the cell body to mediate accelerated axonal regeneration include a reduction in the motoneuron neurofilament expression leading to a reduction of the neurofilament/tubulin synthesis ratio (Tetzlaff et al., 1996). This change in the ratio reduces interaction of neurofilaments with tubulin transport, which, in turn, may allow more tubulin to be transported more rapidly into the growing axon, to support the faster elongation rate of motor axonal regeneration following conditioning lesion. Consistent with this possibility, increases in regeneration rate correlated with an increased in the rate of SCb transport of tubulin

(McQuarrie and Jacob, 1991). The conditioning lesion effect of doubling of regeneration rate in goldfish retinal ganglion cells was associated with increase in both protein synthesis and the velocity and amount of slow axonal transport (McQuarrie and Grafsten, 1982).

The second hypothesis (local growth environment) is supported by the observation that the rate of regeneration in sciatic nerves with the conditioning lesion applied at the same site as the test lesion is greater than in nerves with a more distal conditioning lesion (Bisby and Pollock, 1983; Bisby, 1985). There was no significantly difference in the rate of regeneration whether both sciatic lesions were made at the same site or at 2 different distances from the cell body. Furthermore, when both conditioning and test lesions were made at the same point but far from the cell body, regeneration rate increased more than when the conditioning lesion was proximal to the test lesion and closer to the cell body (Sjöberg and Kanje1990).

In addition, the conditioning lesion effect could not be expressed when the living Schwann cells were removed in the local environment by freezing. Interestingly, the proliferation of Schwann cells in regenerating nerves increases earlier after a test lesion if this lesion was applied at the same site as the conditioning lesion (Sjöberg and Kanje, 1990). "This suggests that not only the neurons but also the Schwann and other non-neuronal cells are conditioned by a nerve lesion".

It is possible that the two hypothetical controls on regeneration rate (cell body changes and the environment of the lesioned axons) may act in an additive fashion to produce a maximal conditioning lesion phenomenon. This idea is supported by the findings of Bisby and Pollock (1983) who reported that nerves with conditioning lesion made distal and prior to the test lesion showed a significant increase in regeneration distance. If the conditioning lesion is made at the same place as the test lesion regardless of the distance between the cell body and the conditioning crush lesion, regeneration distance increased even further.

Recent studies showed that the PMR in the adult rat was enhanced by a conditioning lesion after femoral nerve transection and suture (Brushart, 1998). Normally after adult femoral nerve cut and repair, motor axonal regeneration into motor and sensory pathways is random at 3 weeks, but by 12 weeks, specificity of the regenerating motor axons for the muscle branch is evident (Brushart, 1988). However if the nerve is crushed twice proximal to the repair site 2 and 4 weeks prior to the axotomy and suture, specificity was evident at 3 weeks (Brushart et al., 1998). This specificity was comparable to the amount of specificity seen without a conditioning lesion at 12 weeks. These findings are promising as they suggest that axonal regeneration and generation of specificity can be accelerated in the adult. In addition, they may provide insights into the mechanism(s) of how peripheral nerve regeneration can be induced. However, all the conditioned lesioned studies mentioned above except Brushart et al. (1998) used crush injury rather than transection injury. Furthermore, the need for intervention before nerve injury rules out clinical application.

1.4.3.2 Direct current stimulation (DC electrical field)

Whether or not direct current (DC) stimulation promotes peripheral nerve regeneration remains controversial. There are some reports that indicate that there is no effect of DC field stimulation on the rate of axonal regeneration, regeneration success, or functional outcome (McGinnis and Murphy, 1992; Hanson and McGinnis, 1994). Other studies however, reported that there are a positive effects primarily of increases in neurite growth, orientation of neurites toward the negative pole of an electric field and increase in the number of sprouts (Raman et al., 1987; Kerns et al., 1994; for review, see McCaig and Rajinicek, 1991). The cellular mechanisms underlying these pronounced effects are completely unknown. Recent results from our laboratory however, indicated that DC stimulation has no effect on motor axonal regeneration (Neumann, 1997). The stimulation did not accelerate PMR in adult rat after femoral nerve cut and surgical repair (Neumann, 1997).

1.4.3.3 Alternating current stimulation (electrical stimulation)

Forty-eight years ago Hoffman (1952) first noted the positive effects of neuronal activity on sprouting in partially denervated muscles. He reported that a 1 hr period of electrical stimulation of the spinal cord or nerve roots, delivered immediately after partially denervating the sciatic nerve, accelerated the onset of sprouting from intact axons that normally follows such a partial denervation. He advanced the explanation that electrical stimulation accelerated protein synthesis in the cell body. The phenomenon was later noted in the sympathetic ganglion and in partially denervated skin (Maehlen and Nja, 1982; Doucette and Diamond, 1987).

Maehlen and Nja (1982) reported that preganglionic stimulation at 20 Hz for 1 hr immediately after the partial denervation of the guinea-pig sympathetic ganglion cells increased the rate of sprouting. A very brief natural stimulation of the skin led to a dramatic shortening in the latency of their sprouting into partially denervated skin. This finding that this "precocious" sprouting was prevented by blocking action potential conduction to the cell body with a proximal but not distal tetrodotoxin (TTX) block indicated that the effect of neuronal activity was to cause "a production de novo, or an increased rate of production of some substance(s) in the cell bodies of the activated fibers that must be then conveyed by fast axoplasmic transport to the nerve endings, effectively making these more sensitive to growth factors in the denervated skin" (Doucette and Diamond, 1987). Other studies have also shown that electrical stimulation promotes sprouting and some early functional recovery (Nix & Kopf, 1983; Pockett & Gavin, 1985; Manivannan & Terakawa, 1994). Pockett and Gavin (1985) indicated that 1 hr electrical stimulation of crushed sciatic nerve shortened the latency of the toe spreading reflex. This accelerated regeneration was also observed using the return of twitch force, tetanic tension and muscle action potential amplitude as the outcome variables (Nix and Hopf, 1983).

In summary, electrical stimulation has been shown to promote sprouting and some early functional recovery after nerve crush injuries (for example, Nix & Kopf, 1983; Pockett & Gavin, 1985; Manivannan & Terakawa, 1994). However, effects of electrical stimulation on regeneration after nerve transection have not been evaluated with the exception of preliminary observations from our laboratory (Neumann, 1997). Some PMR was evident 2 weeks after the application of continuous electrical stimulation at 20 Hz to the intact axons in the proximal nerve stump of the cut and surgical repair rat femoral nerve as compared to no PMR at two weeks after the cut and surgical repair and no stimulation. It is the extension of these studies which form the core of this thesis.

1.4.3.4 Nimodipine

Nimodipine which is a L-type Ca^{2+} channel blocker, has been shown to accelerate functional recovery after a crush injury to the sciatic nerve (Zee et al., 1987). Furthermore, Angelov et al. (1996) showed that nimodipine accelerated some axonal sprouting after transection of the facial nerve and suggested that its effect on blood flow is responsible for the acceleration of axonal sprouting. Conversely, recent results from our laboratory indicated that nimodipine has no effect on motor axonal regeneration (Neumann, 1997). Nimodipine did not promote PMR in adult rat after femoral nerve transection and repair (Neumann, 1997). Furthermore, the use of this agent to promote peripheral nerve regeneration after injury could be limited by possible untoward cardiovascular effects (Murad, 1990).

1.4.3.5 FK506

FK506, an immunosuppressant which has been used to prevent allograft rejection in organ transplantations (Hoffman et al., 1990), speeds nerve regeneration in the peripheral nervous system in the rat sciatic crush model (Gold et al., 1994,1995 and 1999, for review, see Gold, 1997). FK506 accelerates functional recovery and rate of nerve regeneration in the adult rat sciatic nerve after crush (Gold et al., 1994,1995 and 1999, for review, see Gold, 1997). FK506 has not yet been shown to promote nerve regeneration after nerve transection until a recent study from our laboratory which demonstrated that FK506 increases the number of motoneurons which regenerated their axons after chronic axotomy but not after chronic denervation (Suilaman et al., 2000). The mechanism by which FK506 promotes nerve regeneration is unknown. Recent study however, indicated that FK506 increases GAP-43 levels in axotomized sensory neurons (Gold et al., 1998) which may play a role in FK506 ability to speed axonal regeneration (Gold et al., 1998). The potential toxicity of FK506 which includes the development of moderate-to-severe neurotoxicity (including cortical blindness, tremor, seizure, and encephalopathy) (Vincenti et al., 1996, for review, see Gold, 1997) could limit its use to promote peripheral nerve regeneration after injury.

1.4.3.6 Neurotrophins

In recent years, neurotrophins have been widely studied in developing neurons and injured neurons. I review here recent cellular and molecular studies which throw some light on what might be done to prevent degenerative changes induced by the nerve injury after axotomy, and to enhance nerve regeneration. In this section I will summarize some of the relevant biology of the effects of neurotrophins on neurons, and review the evidance that neurotrophins can promote peripheral nerve regeneration, concentrating particularly on motoneurons.

The neurotrophins, NGF, BDNF, neurotrophin-3 (NT-3), neurotrophin-4/5

(NT-4/5) and neurotrophin-6 (NT-6), are trophic molecules that play an essential role in the development, maintenance and regulation of neuronal function in both the PNS and the CNS (for review see, Fu and Gordon, 1997; Terenghi, 1999). All 5 neurotrophins support survival of sensory neurons (for review, see Yin et al., 1998). In the CNS, NGF and BDNF both appear to promote the survival and differentiation of cholinergic neurons of the basal forebrain (Alderson et al., 1990). BDNF, NT-3 and NT-4/5 but not NGF, support the survival of dopaminergic neurons of the substantia nigra (Hagg, 1998). Although the effects of neurotrophins on neuronal survival and differentiation have been intensively studied, the evidence that they promote axonal regeneration is, surprisingly, largely indirect and inconclusive. In the PNS, many attempts to promote axonal regeneration by encapsulating neurotrophins in tubes inserted between cut nerves have had limited success with respect to functional outcome. Positive conclusions that the neurotrophins promote axonal regeneration have primarily been based on studies where barely significant differences in regenerated axon counts have been reported (Utley et al., 1996; Lewin et al., 1997).

1.4.3.6.1 Nerve growth factor

NGF was the first growth factor to be described, and is currently the best characterized. So far, two distinct receptors have been identified: a high-affinity tyrosine kinase receptor, trkA and the common neurotrophin receptor p75. NGF supports the survival of embryonic dorsal root ganglion (DRG) sensory neurons and increase the survival of the vast majority of sympathetic and sensory, but not parasympathetic, neurons during the critical stage of development (Deckwerth and Johnson, 1993; for review, see Yin et al., 1998). Immediately after peripheral nerve lesion, there is a decrease of trkA and p75 expression in DRG (Krekoski et al., 1996). The ability of NGF to regulate the expression of neuropeptides differentially in a subset of trkA-expressing DRG sensory neurons is paralleled by its capacity to counteract many degenerative cellular changes induced by the nerve injury (Lindsay and Harmar, 1989; Verge et al., 1996; for review, see Terenghi, 1999). Consistently, administration of exogenous NGF has resulted in enhancement of the initial onset of axonal regeneration from cultured embryonic (Whitworth et al., 1995, 1996) and adult (Lindsay, 1988) DRG sensory neurons, which has been related to a reduction in the incidence of neuronal cell death (Rich et al., 1989). NGF has little or no influence on the survival and the neurite outgrowth of motoneurons (Arakawa et al., 1990; Henderson et al., 1993; Baraun et al., 1996) which do not express trkA receptor (Henderson et al., 1993).

1.4.3.6.2 Brain-derived neurotrophic factor

BDNF displays about 54% homology to NGF. The effects of BDNF are mediated by the tyrosine kinase receptor trkB (for review, see Yin et al., 1998). The binding of BDNF to this receptor leads to autophosphorylation which enables them to bind to and phosphorylate target proteins, so directly activating proteins that induce cell growth and differentiation (for review, see Yin et al., 1998; Terenghi, 1999). Like NGF, BDNF rescues a subset of sensory neurons which express trkB and trkC receptors from naturally occurring cell death (for review, see Yin et al., 1998). BDNF also promotes motor neuron survival in rats (Yan et al., 1992, 1994; Koliatsos et al., 1993). BDNF acts as trophic factor for motoneurons, and in pharmacological doses regulates cholinergic differentiation, supports the survival of motoneurons in culture (Henderson et al., 1993), rescues developing motoneurons from natural cell death (Oppenheim et al., 1992) and prevents the cell death of axotomized motoneurons in anterior spinal horns and facial nucleus (Sendtner et al., 1992; Yan et al., 1992,1994).

That BDNF may play a role in the regeneration of injured adult motoneurons is suggested by the increase in the synthesis of BDNF and its receptors, trkB and p75 by axotomized motoneurons (Ernfors et al., 1989; Meyer et al., 1992; Funakoshi et al., 1993; Piehl et al., 1994; Kobayashi et al., 1996) and the ability of BDNF to promote both phenotypic maintenance after axotomy (Yan et al., 1994) and motor axonal regeneration after chronic motoneuron axotomy (Boyd and Gordon, 2000). Moreover, exogenous application of recombinant human BDNF into the vicinity of axotomized rubrospinal neurons (RSNs), increase the number of axotomized RSNs that regenerate into a peripheral nerve graft concurrent with upregulation of regeneration-associated genes Tα1-tubulin and GAP-43 (Kobayashi et al., 1997).

1.4.3.6.3 Neurotrophin-3

NT-3 is the third member of the newly recognized neurotrophin family. NT-3 shows 55% sequence identity to NGF. NT-3 prefemtially binds to trkC receptor (for review, see Yin et al., 1998). Functionally, NT-3 induces survival and differentiation responses in sensory and parasympathetic neurons, strongly supports the survival of motoneurons in vitro (Henderson et al., 1993), regulates the function of the developing neuromuscular synapses (Lohof et al., 1993), prevents the decline in monosynaptic reflex in 1 a afferent neurons in adult rat after axotomy of the medial gastrocnemius nerve (Mendell et al., 1999) and rescues motoneurons from naturally occurring cell death (Yin et al., 1994). Injection of NT-3 into the lesioned spinal cord increases the regenerative sprouting of the transected corticospinal tract (Schnell et al., 1994). NT-3 and BDNF infusion enhances propriospinal axonal regeneration and more importantly, promotes axonal regeneration of specific distant populations of brain stem neurons into grafts at the mid-thoracic level in adult rat spinal cord (Xu et al., 1995). It would be expected from these results that NT-3 may promote peripheral nerve regeneration.

1.4.3.6.4 Neurotrophin-4/5

A fourth, more distantly related member of the family is neurotrophin-4/5 (NT-4/5). NT-4/5 shows 48% sequence identity to NGF. NT-4/5 binds to the trkB receptor expressed by most rat sensory ganglion cells and motoneurons (Escandon et al., 1994; for review, see Yin et al., 1998). Functionally, NT-4/5 enhances the survival of injured retinal ganglion cells (Sawai et al., 1996), acts as an extremely potent survival factor for motoneurons (Henderson et al., 1993), and attenuates the loss of choline acetyltransferase expression in axotomized motoneurons in adult rats (Friedman et al., 1995). In addition, an in vitro study (coculture model of human skeletal muscle myotubes and rat embryo spinal cord explants) indicates that NT-

4/5 increases the ability of motoneurons to innervate skeletal muscle (Braun et al., 1996). It is reasonable to hypothesize, therefore, that NT-4/5, as well as protecting motoneurons and improving neurite outgrowth, will enhance motor nerve regeneration, but this has not been tested.

1.4.3.6.5 Neurotrophin-6

NT-6 is the most recently-described member of NGF family (Gotz et al., 1994). NT-6 shows 56% sequence identity to NGF. Recombinant purified NT-6 has a spectrum of actions similar to NGF on chick sympathetic and sensory neurons, albeit with a lower potency (Gotz et al., 1994). Its effects on motoneurons deserve to be further investigated.

In summary, the neurotrophins discussed here have been shown to regulate and maintain neuron function, and administration of exogenous neurotrophins counteracts many of the degenerative changes observed in the neurotrophin-responsive subpopulation of axotomized neurons. Unlike NGF itself, the neurotrophins BDNF, NT-3 and NT-4/5 have been shown to have positive effects on motoneurons both *in vitro* and *in vivo*. They attenuate the loss of choline acetyltransferase expression in axotomized motoneurons in adult rats, support the survival of motoneurons *in vitro*, rescue developing motoneurons from naturally-induced cell death, and prevent motoneurons from axotomy-induced cell death *in vivo*. There is reason to think that in the future they may become valuable therapeutic tools, to accelerate motor nerve regeneration and so reduce muscle atrophy. However several aspects of their individual effects remain to be clarified.

1.5 STUDY OBJECTIVES

In the present experiments, we use transected and repaired femoral nerve in the adult rat to investigate motor axonal regeneration after injury and the potential usage of low frequency electrical stimulation to promote the speed and accuracy of motor axonal regeneration. Further experiments were carried out to explore the mechanisms by which electrical stimulation accelerates axonal regeneration and PMR. Understanding these mechanisms might allow us to design therapeutic protocols to improve functional recovery after peripheral nerve injuries.

To avoid ambiguity and to model the most severe clinical scenario, we used an adult rat peripheral nerve (femoral nerve) with complete transection and surgical repair model to answer the following questions: (1) How transection injury of a peripheral nerve affect a) speed and b) accuracy of motor axonal regeneration?, (2) Does low frequency electrical stimulation promote the speed and accuracy of motor axonal regeneration? (3) What is (are) the mechanism(s) of action of electrical stimulation? In the second chapter we describe that 20 Hz electrical stimulation for 1 hr accelerates axonal regeneration and PMR and that this effect is blocked by TTX blockage of action potentials to the cell bodies. In light of the proposed role of BDNF in supporting motoneuronal survival and regeneration along with the rapid and subtle activity-dependent upregulation of BDNF mRNA in central neurons (Zafra et al., 1990, 1991,1992; Castrén et al., 1992,1993; Patterson et al., 1992

Ghosh et al., 1994; Bova et al., 1998 Tao et al., 1998) we investigate whether the positive effect of electrical stimulation is coincident with by upregulation of BDNF and trkB in the stimulated motoneurons. In the final study we determine whether electrical stimulation upregulates the major cytoskeletal proteins, neurofilament and tubulin as well as the growth-associated protein, GAP-43, analogous to the effect of conditioning lesions.

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CHAPTER 2

2.0 BRIEF ELECTRICAL STIMULATION PROMOTES THE SPEED AND ACCURACY OF MOTOR AXONAL REGENERATION

Adapted from the original publication:
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2.1 INTRODUCTION

Injured mammalian peripheral nerves can regenerate over long distances (for review, see Fu & Gordon, 1997). However, it is common clinical experience that functional recovery does not ensue unless transected nerves are surgically repaired to guide regenerating axons into the growth environment of the distal nerve stump (Kline & Hudson, 1995; for review, see Sunderland, 1978). Even then, surgical repair often fails to achieve significant functional recovery, particularly if the injury requires regeneration over long distances and, thus, months and even years of regeneration (Kline & Hudson, 1995; for review, see Sunderland, 1978). These periods are much longer than predicted from reported regeneration rates of 1-3 mm/day, and the latent period of 3-7 days for axons to regenerate across the surgical site and form functional connections (Gutmann et al., 1942; Sunderland, 1947, 1978; Kline & Hudson, 1995).

Axonal regeneration from the proximal stump into inappropriate distal pathways after nerve transection has been long recognised as a factor contributing to poor functional recovery (Langley & Hashimoto, 1917; Kline & Hudson, 1995; for review, see Sunderland, 1978). For example when regenerating motor axons enter Schwann cell tubes in the distal stump that lead to sensory nerve branches, they are directed to sensory end organs. Not only do these axons fail to establish functional contacts, they exclude appropriate axons from entering the pathways which they occupy (Brushart, 1988). However, Brushart (1988, 1993) previously demonstrated that, after femoral nerve transection and repair, motoneurons preferentially reinnervate the quadriceps muscle when given equal access to motor and cutaneous pathways, a process called preferential motor reinnervation (PMR). During early stages of regeneration (2 and 3 weeks), an equal number of motoneurons project correctly to muscle and incorrectly to skin, with many projecting collaterals to both. It is not until later stages of regeneration (8 and 12 weeks) that incorrect collaterals are pruned and the majority of motoneurons project their axons to muscle (Brushart 1988, 1993). Crush proximal to the intended transection site prior to the axotomy and repair produced PMR within 3 weeks (Brushart et al., 1998). These findings are promising as they suggest that the generation of specificity can be accelerated in the adult. However, the need for intervention prior to nerve injury rules out clinical application.

The objective of this study is to determine whether electrical stimulation has the potential to become a viable clinical method for improving functional recovery after nerve transection. Previous studies have shown that electrical stimulation promotes sprouting and some early functional recovery (Nix & Kopf, 1983; Pockett & Gavin, 1985; Manivannan & Terakawa, 1994). However, the effects of electrical stimulation on regeneration after nerve transection have not been evaluated in a comprehensive manner. We have quantified motor axonal regeneration to examine (1) rate of reinnervation of distal nerve stumps after transection injury and (2) whether it is possible to use electrical stimulation to accelerate axonal growth and reinnervation of distal stumps, and to promote the growth of regenerating axons into appropriate pathways.

2.2 MATERIALS AND METHODS

2.2.1 Experimental design

Experiments were performed on the adult rat femoral nerve in which motor axons preferentially reinnervate muscle pathways (Brushart, 1988). The femoral nerve normally contains cutaneous sensory fibers which branch to innervate the skin via the saphenous nerve. These are intermingled with sensory and motor fibers destined for the quadriceps muscle via the quadriceps muscle nerve (Fig. 2.1a). One third of the axons derive from the α-motoneurons which innervate the skeletal muscle fibers (Brushart and Sieler, 1987). Motor axons are found only in the muscle branch so that any motor reinnervation of the sensory branch represents a failure of specificity. Experiments were approved by local ethical committee (Health Science Laboratory Animal Services) under the Canadian guidelines for animal experimentation.

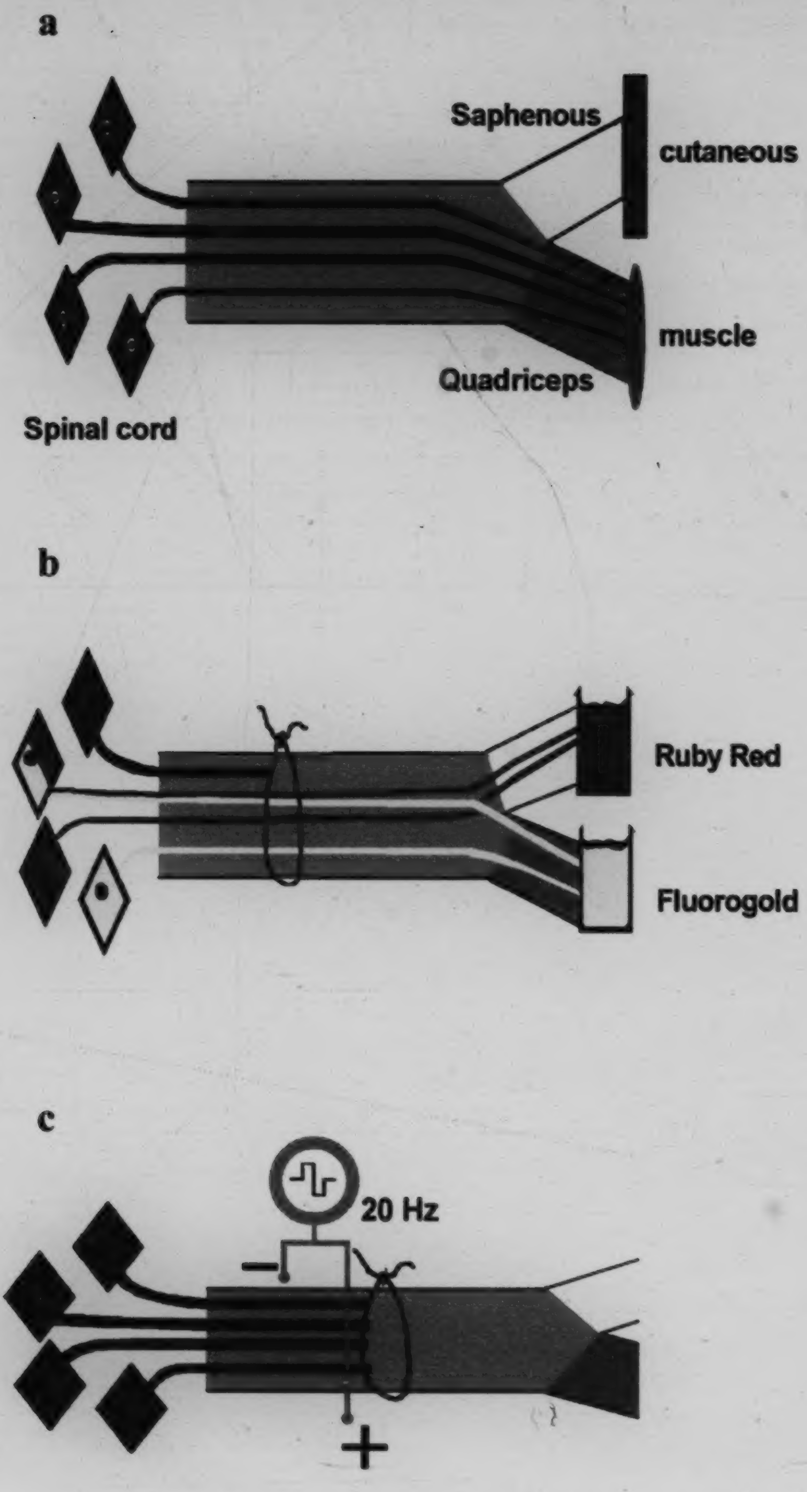
2.2.2 Nerve repair

Experiments were performed under aseptic conditions on the left femoral nerves of young adult (220-240 gm) female Sprague Dawley rats anesthetized with somnotol (30 mg/kg, i.p.). The proximal femoral nerve was sharply cut, 20 mm proximal to the bifurcation into cutaneous and muscle nerves. The proximal and distal stumps were then carefully aligned and surgically joined within a 4 mm long silastic nerve cuff (0.76 mm inner diameter; Dow Corning) by placing a single stitch of 9-0 Ethicon (Ethicon) through the epineurium of the proximal and distal stumps under 40 power magnification (n=45; Fig. 2.1b). Six groups of rats were prepared; regeneration was assessed at 2, 3, 4, 6, 8, and 10 weeks after nerve transection and repair.

2.2.3 Electrical stimulation of axotomized and repaired motoneurons

In experiments in which transected and repaired femoral nerves were electrically stimulated, two insulated Cooner wires (A 5632) were bared of insulation for 2-3 mm and each twisted to form a small loop to secure on either side of the nerve stump proximal to the suture site. The insulated wires were led to a custom-made stimulator which was encased in epoxy and covered with biocompatable silastic. The cathode was sutured alongside the femoral nerve just below its exit from the peritoneal cavity whilst the anode was sutured to muscle close to the nerve, just proximal to the suture repair site. The wires were connected to a custom-made biocompatable implantable stimulator containing a light sensitive diode, which turned the stimulator on and off by an external light flash (Fig. 2.1c). We commenced stimulation immediately after nerve repair with supramaximal pulses (100 μsec, 3V) delivered in a continuous 20 Hz train by the implantable stimulator. We chose a low stimulus frequency of 20 Hz because it is the physiologically relevant frequency of hindlimb motoneuron discharge (Loeb and Hoffer, 1987). In the sham group of rats, the electrodes were implanted but the stimulator was not switched on. The stimulated axotomized motoneurons and their regenerating axons were subject to short-term (1 hr or 1 day) or long-term (1 or 2 weeks) periods of continuous low frequency electrical stimulation (n=95).

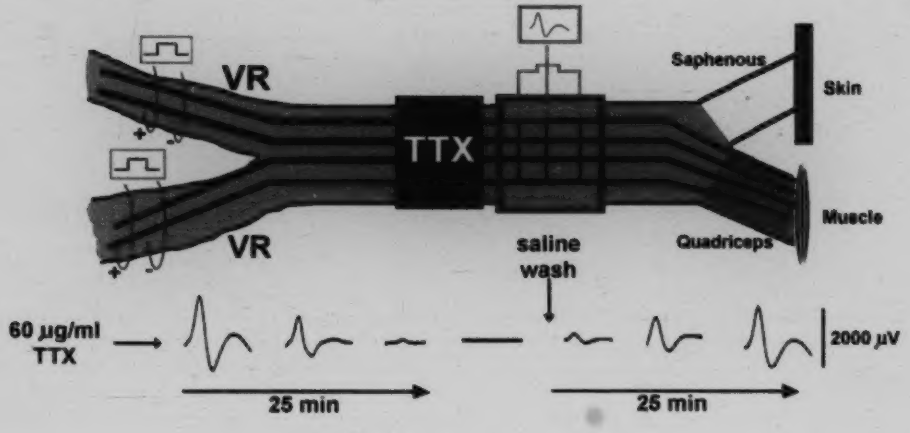
FIGURE 2.1. Diagrammatic representation of the following. **a,** The femoral nerve, the branch to the quadriceps muscles and the saphenous nerve branch containing sensory nerves to the skin. **b,** Application of retrograde neurotracers to count motoneurons which regenerated their axons into the muscle and cutaneous branches of the cut and surgically repaired femoral nerve (see text for details). **c,** Placement of bipolar electrodes to stimulate chronically the cut and regenerating nerve fibers proximal to the site of nerve transection and surgical repair.


a
Saphenous
cutaneous
muscle
Quadriceps
Spinal cord
b
Ruby Red
Fluorogold
c
20 Hz
−
+

2.2.4 Tetrodotoxin application

After determining that electrical stimulation of axotomized and repaired peripheral nerves improved regeneration, we sought to determine whether the effects of electrical stimulation could be mediated via the cell body (see Results). We first determined the blocking doses of tetrodoxin (TTX) in acute in vivo experiments (Fig. 2.2). Under general anesthesia (somnotol 30 mg/kg, i.p.), the femoral nerve was exposed and a laminectomy performed to expose and cut the parent L2 and L3 ventral roots. Ventral roots were maximally stimulated (2 x threshold) via bipolar electrodes to evoke compound action potentials on the femoral nerve 20 mm proximal to the bifurcation point to the muscle and cutaneous saphenous branches. Doses of TTX (30 μg/ml, 60 μg/ml, 120 μg/ml and 240 μg/ml) were applied to the femoral nerve via a Vaseline well placed just outside the peritoneal cavity. The evoked responses on the femoral nerve distal to the TTX blockade were recorded in response to electrical stimulation of the 2 ventral roots at a rate of 20 Hz. Electrical responses were recorded over a time period of 45 min. The effective dose of TTX which blocked action potentials completely and reversibly was 60 mg/ml. We then applied this dose of TTX proximal to the stimulating electrodes prior to the femoral nerve transection, surgical repair and the 1 hr stimulation period. The electrodes and the TTX were removed prior to closing the wound site (n=16).

FIGURE 2.2. Diagrammatic representation of the experimental method used to establish the blocking dose of TTX on the femoral nerve. Bipolar stimulating electrodes placed on each of the 2 ventral roots which supply the motor fibers in the femoral nerve were stimulated supramaximally and the evoked action potential recorded on the femoral nerve distal to the application of TTX to the nerve. Doses of 60 μg/ml were found to be effective in completely blocking action potential conduction. (See the text for further details). *VR*, Ventral root.


VR
VR
+
-
+
-
TTX
Saphenous
Skin
Muscle
Quadriceps
saline wash
60 µg/ml TTX
2000 µV
25 min
25 min

2.2.5 Retrograde labeling of motoneurons

At the end of the regeneration period, the muscle and cutaneous branches of the left femoral nerve were isolated, cut, and backlabeled with neurotracers to identify the motoneurons innervating each branch (Fig. 2.1b). Fluorogold (FG; Fluorochrome Inc., Denver, CO) and Fluororuby (FR; dextran tetramethylrhodamine, D-1817; Molecular Probes, Eugene, OR) were the 2 dyes chosen since they are effectively endocytosed and retrogradely transported (Schmued and Fallon, 1986). The muscle and cutaneous branches were cut 5 mm distal to the femoral bifurcation (25 mm from the repair site). In each rat, one branch was labeled with FG and the other with FR (in practice, the dye application was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes). Backlabeling with FG was done by exposing the tip of the severed branch to 4% FG in 0.1 M cocodylic acid for 1 h in a Vaseline well, after which it was extensively irrigated and reflected to a distant portion of the wound. Backlabeling with FR was done by placing the tip of the severed branch above small weighing paper with FR crystals for 2 h, then irrigating the nerve and placing it in the opposite corner of the wound to prevent cross-contamination by diffusion of tracers. Animals were kept for 72 hr after tracer application in order to allow the retrograde tracers to travel back to the neuronal cell bodies.

2.2.6 Tissue fixation by cardiac perfusion

Rats were deeply anaesthetised (Somnotol, 0.12 ml/100 gm body weight) and perfused through the left ventricle. A warm saline flush (100 ml) was followed by 500 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, over one-half hour. After perfusion, the lumbar spinal cord (T11-L1) which includes all the femoral motoneurons (Brushart & Seiler, 1987) was removed, and post fixed for 1 h in 4% paraformaldehyde, then cryoprotected in 30% sucrose overnight. The tissue were frozen in isopentane cooled to – 70 C° and stored at – 80 C° until further processing (Neumann et al., 1996).

2.2.7 Motoneuron counting

The lumbar spinal cords were cut longitudinally at 50 μm on a freezing microtome (Jung CM 3000). Sections were serially mounted on glass slides, dried, and coverslipped. Each spinal cord section was visualised at 20-40 x under UV fluorescence at barrier filters of 430 nM for FG and 580 nM for FR. Motoneurons containing both FR and FG throughout the cell body were viewed by changing the fluorescent light (Fig. 2.3a). Backlabeled motoneurons were counted by an observer who was unaware of which branch had received FG or FR. The counting of split cells twice was corrected for by the method of Abercrombie (1964). In each group, motoneurons were scored as projecting axons (1) correctly to the muscle branch, (2) incorrectly to the cutaneous branch, or (3) simultaneously to both branches.

2.2.8 Statistical analysis

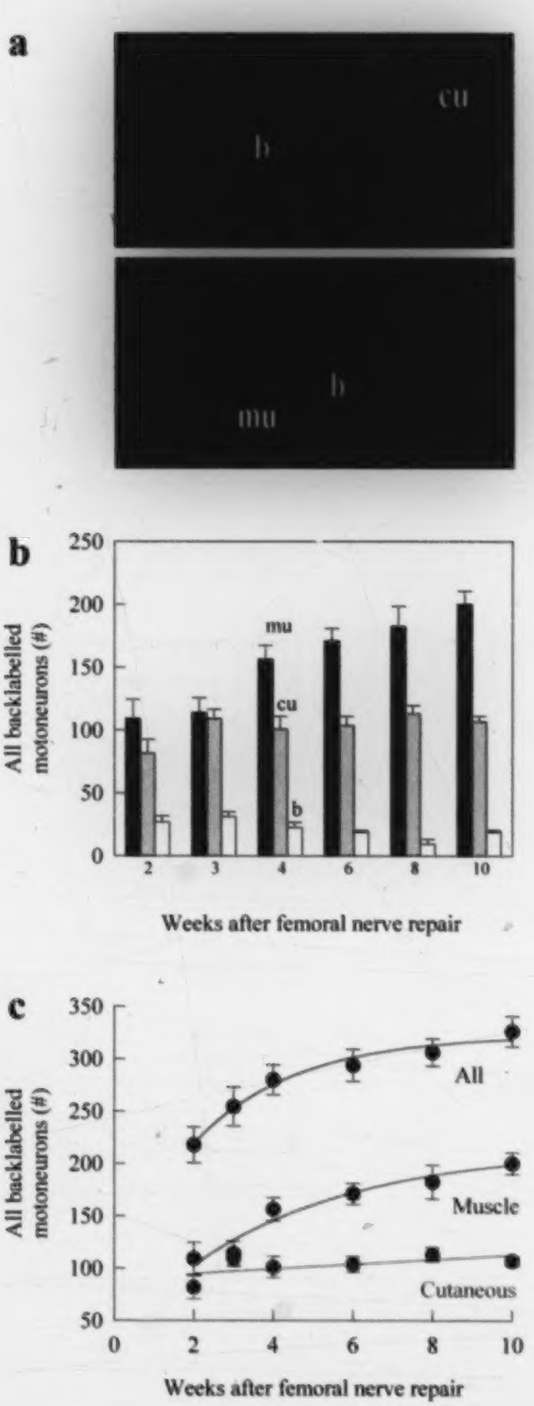
A one-way ANOVA was used to compare the mean number of motoneurons projecting axons to cutaneous and muscle branches within each group. A multi-factorial ANOVA was used to compare the mean number of motoneurons projecting axons to cutaneous, muscle, and both branches among all groups. The same comparison was used for the stimulation, sham-stimulation, and TTX groups. Statistical significance was accepted at the 0.05 level.

2.3 RESULTS

2.3.1 Emergence of PMR associated with staggered regeneration

At the established regeneration rate of 3 mm/day (Gutmann et al., 1942), axons might be expected to regenerate over a distance of 25 mm in the course of 2 and, at most, 3 weeks. However, some regenerating femoral motor axons did not reach a point 25 mm from the site of surgical repair until 8 or 10 weeks later (Fig. 2.3b,c). Axons thus cross the repair site and/or regenerate at different speeds. This staggered progressive reinnervation was associated with progressive preferential reinnervation of appropriate muscle pathways by the regenerating motor axons. Two and 3 weeks after nerve repair, an equal number of motoneurons regenerated their axons into appropriate and inappropriate muscle and cutaneous pathways, respectively (Fig. 2.3b,c). In addition, a small but significant number regenerated axon collaterals into both branches, identified as being double labeled (Fig. 2.3a-c). However, between 3 and 4 weeks the number of motoneurons with correct projections to the muscle branch increased significantly, with little change in the number which regenerated their axons into the cutaneous branch. As a result, the difference between the mean ($\pm$SE) number of motoneurons which regenerated their axons into muscle and cutaneous branches became statistically significant ($p < 0.05$) (Fig. 2.3b,c). When the backlabeling was carried out at 6, 8 and 10 weeks after repair, there was a progressive increase in the number of motoneurons which regenerated specifically into the muscle branch, while the number in the inappropriate cutaneous branch apparently remained static (Fig. 2.3b). Emergence of PMR thus occurs between 3 and 4 weeks and becomes very distinct by 10 weeks. A small proportion of these were accounted for by a small decline in double labeled motoneurons, the interpretation being that axonal collaterals in the "wrong" cutaneous branch are withdrawn or pruned (Brushart, 1988, 1993). Thus emergence of PMR occurs primarily as a result of the progressive regeneration of motor axons specifically into the appropriate muscle branch between 2 and 10 weeks. The number of motoneurons which regenerated into the inappropriate cutaneous branch did not change after 2 weeks, presumably because the motoneurons which regenerated thereafter are directed specifically into the appropriate muscle branch. Eight to 10 weeks were required for all injured motoneurons to regenerate their axons into distal branches. The mean number at 10 weeks (326 ± 14) was not significantly different than the number of the intact contralateral femoral motoneurons (338 ± 8)

Figure 2.3. Counting of the number of femoral motoneurons which regenerated their axons into the appropriate muscle branch and into the inappropriate cutaneous sensory branch, and those which regenerated axons into both. **a,** Retrogradely labeled motoneurons which had regenerated their axons into the appropriate muscle branch (*mu*; *fluororuby*), the inappropriate cutaneous sensory branch (*cu*; *fluorogold*) and those which regenerated axons into both (*b*; *double labeled*). **b,** The mean number of backlabeled motoneurons which regenerated into the appropriate muscle branch (*mu*; *filled bars*), the inappropriate cutaneous branch (*cu*; *stripped bars*), and both branches (*b*; *open bars*) 2, 3, 4, 6, 8, and 10 weeks after femoral repair. **c,** The mean number ±S.E. of total backlabeled motoneurons (*All*) which regenerated their axons into the appropriate muscle branch (*Muscle*) and into inappropriate cutaneous branch (*Cutaneous*) as function of time after femoral nerve transection and repair.


a
cu
b
b
mu
b
mu
cu
b
All backlabelled motoneurons (#)
250
200
150
100
50
0
2
3
4
6
8
10
Weeks after femoral nerve repair
c
All backlabelled motoneurons (#)
350
300
250
200
150
100
50
0
2
4
6
8
10
All
Muscle
Cutaneous
Weeks after femoral nerve repair

2.3.2 Short- and long-term electrical stimulation are equally effective in accelerating regeneration and PMR

The effects of electrical stimulation of the transected and surgically repaired femoral nerve were dramatic. We initially chose to stimulate for a 2 week period because motor axonal regeneration into cutaneous and muscle branches is equal 2 weeks after nerve repair in this model (Brushart, 1988,1993; Neumann et al., 1996) (Fig. 2.3b). We found that 2 weeks of electrical stimulation at 20 Hz accelerated axonal regeneration, such that all motoneurons regenerated their axons within 3 weeks, in contrast to the 8-10 weeks required without stimulation (Fig. 2.4).

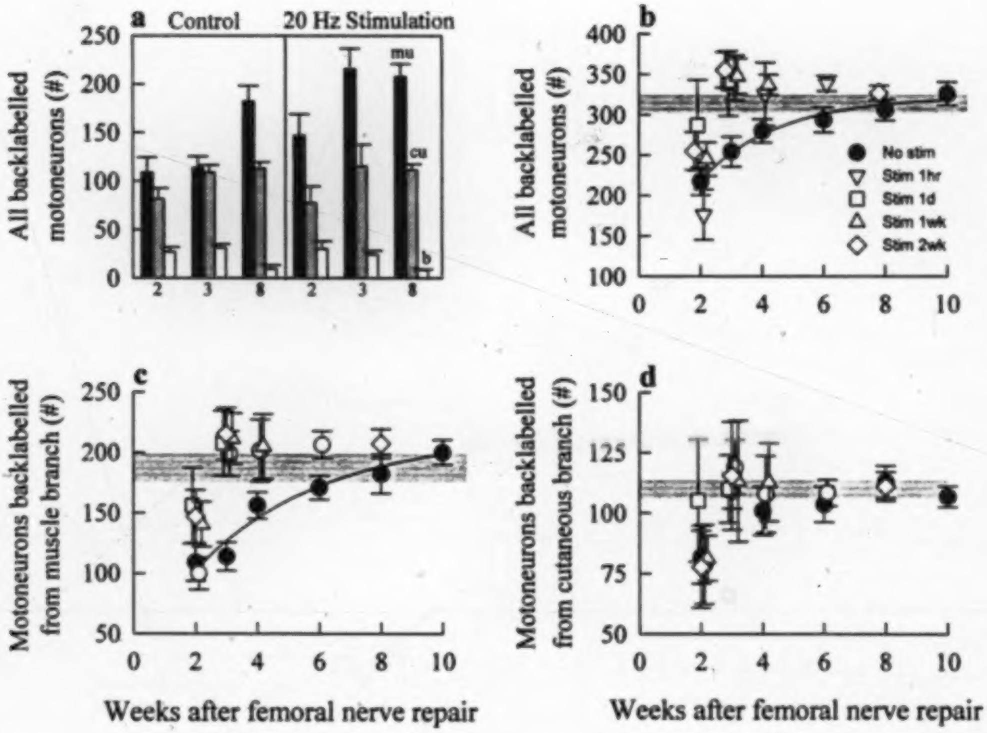
Acceleration of axonal regeneration by electrical stimulation was accompanied by accelerated preferential growth of these regenerating axons into appropriate muscle pathways and not into inappropriate cutaneous pathways (Fig. 2.4a). The dramatic acceleration of both axonal regeneration and PMR by electrical stimulation is clearly seen when the total number of motoneurons which regenerated and those which regenerated into the muscle and cutaneous branches are plotted as a function of time after femoral nerve section and surgical repair (Fig. 4b-d). As shown by the *open diamonds*, 2 weeks of electrical stimulation promoted axonal regeneration of all injured motoneurons by 3 weeks, as compared to the normal 8-10 week period required for all motoneurons to regenerate 25 mm from the suture site (Fig. 2.4b). The increase in the total number of motoneurons regenerating is accounted for by a corresponding increase in the number which regenerated axons into the appropriate muscle branch (Fig. 2.4c). The number of motoneurons which regenerated their axons into the sensory branch did not increase significantly between 2 and 10 weeks. Noticeably however, the variability in this number decreased substantially as a function of time (Fig. 2.4d). Although there was also a trend for the number of double labeled motoneurons to decline with stimulation, the numbers were not statistically different ($p > 0.05$).

To optimise the potential utility of electrical stimulation for clinical nerve repair, we progressively reduced the duration of low-frequency continuous electrical stimulation from 2 weeks to 1 hr. Whether the proximal nerve stump was electrically stimulated at 20 Hz for a 2 week period or for as little as 1 hr (Fig. 2.4), the stimulation accelerated axonal regeneration so that all axotomized motoneurons regenerated by 3 weeks (Fig. 2.4b). This is well illustrated by comparing the effectiveness of short- and long-term electrical stimulation relative with the effects of sham stimulation on the number of motoneurons which were backlabeled from the muscle and cutaneous sensory nerve branches 3 weeks after cutting and repairing the femoral nerve (Fig. 2.5). We made the comparison at 3 weeks after nerve repair, at a time when the number of motoneurons which have regenerated is still ~50% of the total, and PMR has not emerged in sham-stimulated femoral nerves. The histograms in figure 2.5 demonstrate that short- and long-term stimulation were equally effective in increasing the number of motoneurons which regenerated their axons into the muscle branch without affecting the number that regenerated into the inappropriate cutaneous branch.

The dramatic effect of electrical stimulation in accelerating regeneration and

PMR is consistent from animal to animal (Fig. 2.5b,c). The contrast between the number of motoneurons which regenerated axons in the distal nerve branches after 1 hr stimulation compared to sham-stimulation demonstrates: (1) incomplete axonal regeneration and lack of preferential motor reinnervation, 3 weeks after femoral nerve transection and repair without stimulation, and (2) the effectiveness of electrical stimulation in increasing the number of motoneurons which regenerated their axons over a 25 mm distance. The consistency between individual animals is particularly striking in the context of the inherent variability of surgical procedures (Fig. 2.5b,c). Our data thus demonstrate that we can reduce the duration of continuous low-frequency stimulation and still accelerate motor axonal regeneration in the appropriate muscle pathways to result in preferential motor reinnervation.

FIGURE 2.4. Effects of electrical stimulation on motor axonal regeneration and PMR. **a,** The mean number ±S.E. of motoneurons which regenerate into appropriate muscle (*mu*, *filled bars*) and inappropriate cutaneous (*cu*, *stripped bars*) branches and both branches (*b*, *open bars*) 2, 3 and 8 weeks after femoral nerve transection and surgical repair and 2 weeks 20 Hz continuous electrical stimulation. **b-d,** Comparison of the mean ±S.E. of the numbers of motoneurons which regenerated after femoral nerve transection and surgical repair without (●) and with 20 Hz continuous electrical stimulation for 1 hr (▽), 1 d (□), 1 week (△), and 2 weeks (◇). **b,** All motoneurons. **c,** Motoneurons which regenerated into the appropriate muscle branch. **d**, Motoneurons which regenerated into the inappropriate cutaneous branch. The *shaded horizontal bar* in b-d represents ±S.E. of the mean number of regenerated motoneurons 8 and 10 weeks after femoral nerve repair with no stimulation, when there was no longer any significant difference between mean numbers of regenerated motoneurons ($P > 0.05$). *stim*, Stimulation.


a
Control
20 Hz Stimulation
All backlabelled motoneurons (#)
250
200
150
100
50
0
2
3
8
2
3
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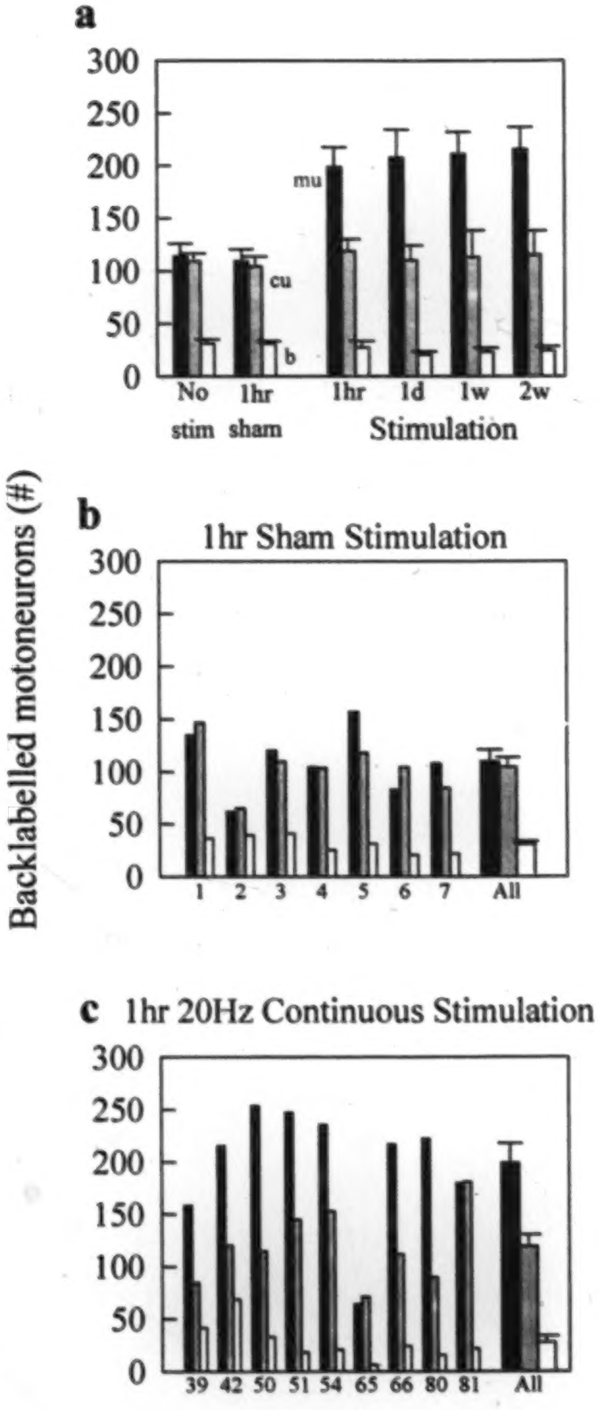
cu
b
b
400
350
300
250
200
150
100
All backlabelled motoneurons (#)
0
2
4
6
8
10
No stim
Stim 1hr
Stim 1d
Stim 1wk
Stim 2wk
c
Motoneurons backlabelled from muscle branch (#)
250
200
150
100
50
0
2
4
6
8
10
Weeks after femoral nerve repair
d
Motoneurons backlabelled from cutaneous branch (#)
150
125
100
75
50
0
2
4
6
8
10
Weeks after femoral nerve repair

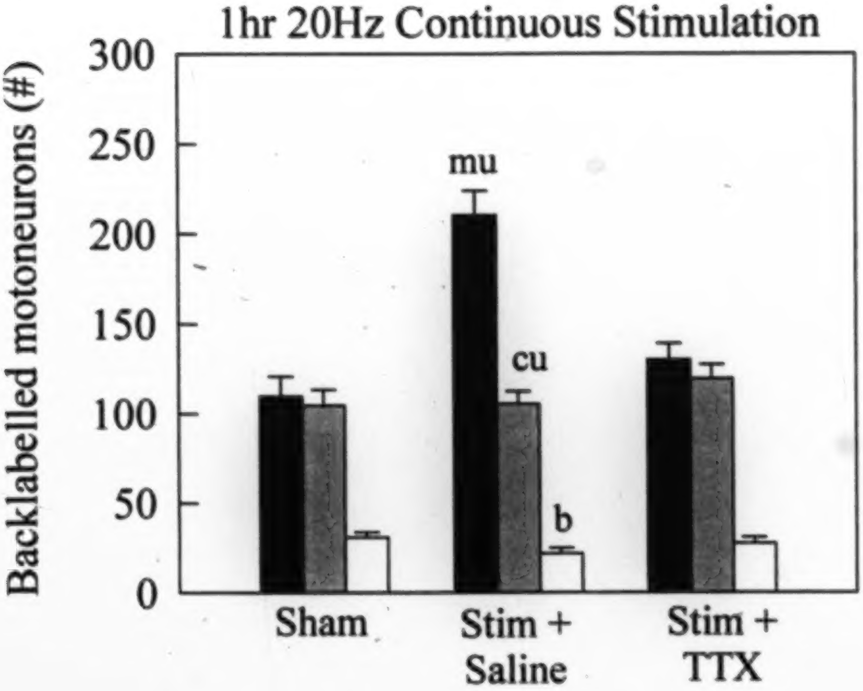
FIGURE 2.5. Short-term stimulation is as effective as long-term stimulation in accelerating axonal regeneration and PMR. **a,** Comparison of effects of different periods of 20 Hz continuous electrical stimulation (1 hr, 1 d, 1 week and 2 weeks) on the mean ±S.E. number of motoneurons which regenerated into muscle (*mu*; *filled bars*), cutaneous (*cu*; *stripped bars*) and both (*b*; *open bars*) branches of the femoral nerve 3 weeks after nerve repair as compared with no stimulation or sham-stimulation. **b, c**, Data from individual rats (numbered on the *x*-axis) are shown the effects of 1 hr sham-stimulation (**b**) and 1 hr 20 Hz continuous electrical stimulation of proximal nerve stump immediately after nerve repair (**c**). *stim*, Stimulation.


a
300
250
200
150
100
50
0
mu
cu
b
No stim
1hr sham
1hr
1d
1w
2w
Stimulation
Backlabelled motoneurons (#)
b
1hr Sham Stimulation
300
250
200
150
100
50
0
1
2
3
4
5
6
7
All
c
1hr 20Hz Continuous Stimulation
300
250
200
150
100
50
0
39
42
50
51
54
65
66
80
81
All

2.3.3 The positive effect of short-term electrical stimulation is mediated via the cell body

The effectiveness of only 1 hr stimulation of the axotomized motoneurons suggested that the site of action of the electrical stimulation could be the cell body, possibly by initiating the growth program earlier. To test this hypothesis, we blocked the retrograde transmission of action potentials to the cell body as well as the afferent evoked anterograde excitation of the motoneurons using a TTX block of sodium channels. We found that the TTX blockade completely prevented the effect of the 1 hr stimulation (Fig. 2.6). These experiments localise the site of action of the electrical stimulation to the cell body.

FIGURE 2.6. TTX block of retrograde transmission of action potentials to the cell body. TTX (60 µg/ml) completely blocked the effect of 1 hr 20 Hz continuous electrical stimulation in accelerating regeneration and PMR 3 weeks after nerve transection and surgical repair. *b* (*open bars*), Both branches; *cu* (*stripped bars*), cutaneous branch; *mu* (*filled bars*), muscle branch; *stim*, Stimulation.


1hr 20Hz Continuous Stimulation
Backlabelled motoneurons (#)
300
250
200
150
100
50
0
mu
cu
b
Sham
Stim + Saline
Stim + TTX

2.4 DISCUSSION

In this study, we used an adult rat peripheral nerve transection and surgical repair model to demonstrate that axons continue to reinnervate the distal pathway for protracted periods of up to 10 weeks. At a regeneration rate of 3 mm/day, many axons which would be expected to regenerate over a distance of 25 mm in 2-3 weeks, did not do so until 8 to 10 weeks had elapsed. This process of gradual or staggered reinnervation is associated with progressive reinnervation of appropriate muscle pathways by regenerating motor axons (PMR). We have also demonstrated that both the staggered axonal regeneration and PMR can be accelerated by electrical stimulation of the axotomized motoneuron. This positive effect of electrical stimulation is mediated at the cell body and requires as little as 1 hr of electrical activity.

2.4.1 Staggered axonal regeneration

When the femoral nerve was cut and surgically reunited, the number of motoneurons which regenerated their axons over a 25 mm distance increased to a maximum by 8-10 weeks after repair (Fig. 2.3). Since this number equalled the total number of motoneurons which supplied the intact femoral nerve in the contralateral leg, it is evident that all motoneurons eventually regenerated their axons across the suture line. However, this process occurred gradually over a 10 week period. The speed of axonal regeneration has classically been determined by measuring the distance from the injury site at which a pinch stimulus evokes a response (Young & Medawar, 1940). The widely reported rate of regeneration of 3 mm/d (Gutmann et al., 1942; Seddon et al., 1943; Sunderland, 1947; for review, see Sunderland, 1978) thus describes only the outgrowth of the fastest growing sensory axons. The prolonged period over which axons regenerate across a surgical repair would thus evade detection. While this prolonged period has long been suspected on the basis of clinical experience (Kline & Hudson, 1995; for review, see Sunderland, 1978), this is the first experimental quantification of the prolonged duration of axonal regeneration after a surgical cut and repair. A distance of 25 mm is traversed by waves of regenerating motor axons which arrive at the point of backlabeling at widely different times. Interestingly, this finding is quite consistent with and provides a clear explanation for "unpublished findings of an unusually broad peak of axonally transported radioactivity in regenerating nerves after transection injuries" that was reported in the paper of Forman & Berenberg (1978).

Because the fastest axons regenerate at a rate of 1-3 mm/d, delays of days and weeks must occur before many axons enter the distal nerve stumps, or as they propagate within it. The former possibility was suggested by the drawings of Ramon Y Cajal (1928) of the tortuous pathways taken by growth cones crossing from proximal to distal nerve stumps. Characteristically, axonal sprouts emerge from the first node of Ranvier proximal to an injury with many axon collaterals (5-20) entering the distal nerve stump (Morris et al., 1972; Mackinnon et al., 1991). Nonetheless, sprouts may bud from even more proximal nodes (Mackinnon et al.,

1991) which could have the effect of staggering regeneration. In addition, it is possible that the outgrowth of many daughter axon collaterals from the parent axon slows axonal regeneration. Perhaps axons with multiple sprouts grow slowly until enough sprouts are pruned to allow routing of most structural materials to the dominant sprout which, thereafter, regenerates more rapidly. It is conceivable that axon collaterals from a motoneuron regenerated into both muscle and cutaneous nerve branches but that, at the time of backlabeling at 2 weeks and a distance of 25 mm from the site of entry of the regenerating axons into muscle or cutaneous pathways, most of the inappropriate collaterals were removed or pruned so rapidly that they were not detected in the wrong pathways as double-labeled motoneurons or as motoneurons with axons in the sensory branch.

2.4.2 Electrical stimulation and accelerated axonal regeneration

Electrical stimulation dramatically accelerated axonal regeneration. Electrical stimulation, applied immediately after surgical repair of the cut femoral nerve promoted the regeneration of all motor axons over a 25 mm distance from the surgical site in 3 weeks. The regenerating axons required 8-10 weeks to reach this level without stimulation (Fig. 2.3b,c). This finding substantiates the conclusions that electrical stimulation can accelerate axonal regeneration which were drawn from experiments which detected slightly earlier and larger electromyographic signals and accelerated recovery of force in reinnervated rabbit soleus muscles after crush injury (Nix & Kopf, 1983). The acceleration of axonal regeneration is much more dramatic in our study, probably because (1) the nerve section injury prolonged the period of axonal outgrowth from the proximal nerve stump more than the crush injury and (2) there are substantial delays associated with reformation of nerve-muscle connections which are avoided by determining the number of motoneurons which regenerated into the distal nerve stump.

2.4.3 Preferential motor reinnervation

Findings that femoral motor axons regenerated equally into the appropriate muscle and cutaneous branches of the nerve 2-3 weeks after nerve repair, are quite consistent with previous findings of Brushart (1988, 1993). With continued regeneration, preferential reinnervation of the muscle nerve (PMR) was demonstrated at 8 and 12 weeks. Our present study evaluated this process at more frequent intervals (2, 3, 4, 6, 8, and 10 weeks). The progressive increase in correct projections from week 4 to week 10 paralleled a large increase in the total number of motoneurons regenerating (Fig. 2.3b,c). Hence PMR emerges with time concurrent with a progressive increase in the number of motoneurons which regenerate their axons into the distal nerve stumps. Differences between the number of motoneurons which regenerated into the muscle as compared to the cutaneous nerve branches became more prominent as the number of motoneurons which participated in the regeneration increased. It was particularly striking that emergence of the PMR was directly associated with the increased numbers of motoneurons which regenerated axons specifically into the muscle nerve as opposed to the cutaneous nerve. In fact, the mean number of motoneurons (120) which had regenerated their axons inappropriately into the cutaneous nerve branch remained unchanged from 2 weeks onward. This finding suggesting that a mechanism capable of directing highly specific regeneration becomes activated 3 weeks after repair. The presence of the L2/HNK-1 carbohydrate in muscle but not in cutaneous pathways and its selective upregulation by regenerating motor axons suggest that it may participate in this process (Martini et al., 1994). Although there was a small decline in the number of motoneurons which regenerated into both muscle and cutaneous branches with time, the "pruning" is a relatively minor contributor to the emergence of PMR in adult rat nerve regeneration as compared to juvenile (cf. Brushart 1990, 1993).

2.4.4 Electrical activity accelerates PMR

Electrical stimulation accelerated both axonal regeneration and the development of PMR. The difference between the number of motoneurons projecting to muscle and cutaneous branches of the femoral nerve normally seen 8-10 weeks after nerve repair, was present after only 3 weeks (Fig. 2.4a). This dramatic effect of electrical stimulation mimicked the effects of a proximal crush in accelerating PMR (Brushart et al., 1998). Interestingly, under both conditions, the PMR was associated with increased numbers of motoneurons which regenerated their axons into the distal nerve stump [compare the present study, Fig. 2.4a, with Brushart et al., 1998, their Fig. 2].

2.4.5 Electrical stimulation accelerates axonal regeneration and PMR via the cell body

Tetrodotoxin blockade of action potentials to the cell body abolished the effects of electrical stimulation on the speed and the specificity of motor axonal regeneration. This suggests that electrical stimulation produces its effects at the level of the cell body. These findings are consistent with *in vitro* evidence for depolarization-induced calcium entry into the cell body which is associated directly with upregulation of immediate early genes, initiation of gene expression and neurite outgrowth (Kocsis et al., 1994). One possibility is that electrical stimulation may mediate its positive effect on regeneration by enhancing the cell body response which, in turn, is partially attributed to an enhanced production of the neurotrophin, brain-derived neurotrophic factor (BDNF) and its high affinity receptor TrkB. BDNF and TrkB mRNA and protein levels are elevated after axotomy of motoneurons (Meyer et al., 1992; Funakoshi et al., 1993; Piehl et al., 1994; Kobayashi et al., 1996; for review, see Fu & Gordon, 1997). BDNF has also been shown to promote phenotypic maintenance after motoneuron axotomy (Yan et al., 1994). Moreover, the expression of BDNF and TrkB has been shown to be regulated by neuronal activity (e.g. Zafra et al., 1990, 1991; Tao et al. 1998) and physiological stimuli (e.g. Castren et al. 1992).

2.4.6 Significance

One hr of electrical stimulation dramatically accelerates both axonal regeneration and PMR in the adult rat femoral nerve transection and repair model. Both of these effects have the potential for clinical application. Acceleration of regeneration would counteract the delay in reinnervation of pathways and end organs which compromise functional outcome (see Fu & Gordon, 1995a,b; 1997). Augmentation of PMR could improve recovery after injuries to nerve trunks containing both cutaneous and muscle axons. One hr of stimulation can easily be applied during peripheral nerve surgery. The promising and potential usefulness of the technique clearly warrant further investigation.

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CHAPTER 3

3.0 ELECTRICAL STIMULATION ACCELERATES AND INCREASES EXPRESSION OF BDNF AND TRKB RNA IN REGENERATING RAT FEMORAL MOTONEURONS

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3.1 INTRODUCTION

The neurotrophins are structurally and functionally related polypeptides that support the development, maintenance and plasticity of distinct populations of central and peripheral neurons (for review, see Davies, 1994; Lindholm et al., 1994; Lindsay et al.,1994; Sendtner et al., 1994; Thoenen, 1995; Fu and Gordon, 1997; Terenghi, 1999). Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family, which also includes nerve growth factor (NGF), neurotrophin-3 (NT-3), NT-4/5 and NT-6 NT-7 (for review, see Fu and Gordon, 1997; Yin et al., 1998; Terenghi, 1999).

Motoneurons are equipped to respond to several neurotrophins. They express the signal transducing receptor for BDNF and NT-4, trkB, the signal transducing receptor for NT-3, trkC (for review, see Chao, 1992),and the low-affinity (common) neurotrophin receptor, p75. The latter is expressed during development and in the adult after axotomy (Ernfors et al., 1989). BDNF, NT-3 and NGF are retrogradely transported by motoneurons (DiStefano et al., 1992), and the transport is augmented by neuronal injury (Curtis et al., 1998). BDNF, and to some extent NT-3, promote motoneuron survival both *in vivo* (Oppenheim et al., 1992; Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993) and *in vitro* (Henderson et al., 1993; Hughes et al., 1993). That BDNF may play a role in the regeneration of injured adult motoneurons is suggested by the increase in BDNF and trkB receptor synthesis by axotomized motoneurons (Meyer et al., 1992; Funakoshi et al., 1993; Piehl et al., 1994; Kobayashi et al., 1996) and the ability of BDNF to promote both phenotypic maintenance after axotomy (Yan et al., 1994) and motor axonal regeneration after chronic motoneuron axotomy (Boyd and Gordon, 2000).

Regulation of BDNF expression in neurons is clearly linked to electrical activity. Activation of L-type voltage sensitive Ca^{2+} channels or the non N-methyl-D-aspartate (NMDA) subtype of glutamate receptors leads to an enhancement of BDNF mRNA levels in hippocampal neurons (Zafra et al., 1990, 1991) and in cortical neurons (Ghosh et al., 1994; Tao et al., 1998). In addition, stimuli that evoke long-term potentiation (LTP) *in vivo* (Castrén et al., 1993) and *in vitro* (Patterson et al., 1992) induce BDNF mRNA expression in hippocampal neurons. Moreover, several studies have shown that BDNF mRNA levels are also induced by physiological stimuli (Castrén et al., 1992; Bova et al., 1998).

We have recently reported that continuous electrical stimulation at 20 Hz greatly promotes speed and accuracy of motor axonal regeneration after femoral nerve cut and repair (Al-Majed et al., 2000) (Chapter 2). The rapid and subtle activity-dependent upregulation of BDNF mRNA in central neurons, along with the proposed role of BDNF in supporting motoneuronal survival and regeneration, suggests that BDNF may mediate the effects of stimulation in accelerating motor axonal regeneration. In this paper we have used semi-quantitative *in situ* hybridization (ISH) to investigate the hypothesis that electrical stimulation accelerates motor axonal regeneration by inducing rapid expression of motoneuronal BDNF and trkB.

3.2 MATERIALS AND METHODS

Twenty-eight young adult (220-240 gm) female Sprague Dawley rats were used for the experiments. All surgical procedures were performed under aseptic conditions on animals deeply anesthetized with somnotol (30 mg/kg, i.p.). Experiments were approved by the local ethics committee (Health Science Laboratory Animal Services) under the guidelines of the Canadian council for animal care.

3.2.1 Femoral nerve model

Experiments were performed on the adult rat femoral nerve in which cut motor axons regenerate in a staggered manner and preferentially reinnervate muscle nerve pathways (Al-Majed et al., 2000) (chapter 2). The femoral nerve normally contains cutaneous sensory fibers that innervate the skin via the saphenous nerve. These are intermingled with sensory and motor fibers destined for the quadriceps muscle via the quadriceps muscle nerve [(Fig.1a in Al-Majed et al., 2000 (chapter 2)]. One third of the axons derive from α-motoneurons which innervate the skeletal muscle fibers (Brushart & Seiler, 1987).

3.2.2 Retrograde labeling of motoneuron pools

Two different fluorescent neurotracers were used to label the motoneuron pools of the quadriceps muscles of the left and right hindlimb prior to femoral nerve repair (Fig. 1). Fluorogold (FG; Fluorochrome Inc., Denver, CO) and fluororuby (FR;

dextran tetramethylrhodamine, D-1817; Molecular Probes, Eugene, OR) were chosen because they are effectively endocytosed and retrogradely transported (Laurence & James, 1986). In addition, the 2 dyes are visualised under 2 different fluorescent filters which display the backfilled motoneurons in 2 clearly different colours (Al-Majed et al., 2000) (Chapter 2). This allows us to discriminate between the injured and intact motoneurons on either side of the cord. A small skin incision of less than 1 mm in length was made over the belly of the quadriceps muscle for microinjection of the tracers. Fluorogold (7% in DMSO-saline) or FR (10% in DMSO-saline) was then injected into the belly of the quadriceps muscle (Richmond et al., 1994). The effectiveness of this technique in labeling femoral motoneurons was first assessed in intact nerve muscle preparations of 4 rats. Approximately 18 μl of the tracer was delivered over the course of 5 minutes to each muscle using a 26-gauge needle connected to a Hamilton microsyringe. The needle was advanced across the tendinous midsection separating the medial and lateral compartments and withdrawn slowly during the injection to leave a track of injected material across the 2 compartments close to its innervation band (Richmond et al. 1994). After careful retraction of the syringe to avoid leakage of tracer, the injection site was cleaned and the skin sutured. In each rat, one muscle was labeled with FG and the other with FR (in practice, the dye application was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes). Two weeks after muscle injection of the neurotracers, rats were deeply anaesthetised (Somnotol 0.12 ml/100 gm of body weight) and the spinal cord was fixed with 4%

paraformaldehyde by perfusing through the left ventricle. A warm saline flush of 100 ml was followed by 500 ml of ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) over one-half hour. Thereafter, the lumbar spinal cord (T11-L1) containing the femoral motoneurons (Brushart & Seiler, 1987) was removed, post-fixed for 1 hr in 4% paraformaldehyde, and cryoprotected in 30% sucrose overnight. The tissues were frozen in isopentane, cooled to – 70 C°, and stored at – 80 C° until further processing (Neumann et al., 1996).

The lumbar spinal cords were cut longitudinally at 50 μm on a freezing microtome (Jung CM 3000). Sections were serially mounted on glass slides, dried, coverslipped (Cytoseal; Stephens Scientific), and inspected for the labeled motoneurons at 20-40 x under a UV fluorescence microscope (Leitz, Wetzlar, Germany) using barrier filters of 430 nM for FG and 580 nM for FR. Backlabeled motoneurons were counted by an observer who was unaware of which muscle had received FG or FR. The counting of split cells twice was corrected for by the method of Abercrombie (1964)

3.2.3 Nerve repair

Nerve repair was performed 2 weeks after injection of tracers into the muscles to avoid lesion effects on motoneuron gene expression. In 24 rats, motoneurons were backlabeled by intramuscular injection of FG or FR. Two weeks later, the left proximal femoral nerve was sharply cut 20 mm proximal its bifurcation into cutaneous and muscle nerves. The proximal and distal stumps were then carefully aligned and surgically joined within a 4 mm long silastic nerve cuff (0.76 mm inner diameter; Dow Corning) by placing a single stitch of 9-0 Ethicon (Ethicon) through the epineurium of the proximal and distal stumps under 40 power magnification [(cf. Fig. 2.1b in Al-Majed et al., 2000) (Chapter 2)]. Electrodes were placed as described by Al-Majed et al. (2000) (Chapter 2) proximal to the repair site immediately after nerve repair for supramaximal continuous stimulation (100 μs, 3 V) of the proximal nerve stump at 20 Hz for 1 hr (experimental group; n=12) or sham-stimulation with stimulators off (n=12). During the 1 hr period, the injury site was kept moist by covering the exposed tissues with a saline moistened gauze. Subsequently the electrodes were removed, the wound was closed and the skin was sutured. Groups of 4 stimulated and 4 sham-stimulated rats were then allowed to survive for 8 hr, 2 d, or 7 d after nerve transection and repair. The rats were then killed by an overdose of somnotol and the lumbar spinal cord (T11-L1) was removed immediately, fresh frozen in isopentane cooled to – 70 C°, and stored at – 80 C° until further processing to prevent RNA breakdown by RNAases.

3.2.4 *In situ* hybridization

The fresh-frozen lumbar spinal cords (~1500 μm in length) were sectioned in the coronal plane at 12 μm on a freezing microtome (Jung CM 3000). Each spinal cord tissue section contained both left (axotomized) and right (contralateral control) motoneurons. Superfrost Plus slides (Fisher Scientific; Pittsburgh, PA) were used to collect 6 serial sections. The process was repeated 6 times on each slide, so that neighbouring sections on each slide were 72 µm apart, eliminating the possibilities of repetitive sampling of motoneurons. Eighteen-20 slides per animal were collected. Slides were stored at –80 C°.

In situ hybridization was carried out under RNAase free conditions. Synthetic oligonucleotide probes were used for ISH as described by Verge et al. (1992). In brief, we used a 50 mer oligonucleotide 5'-AGTTCCAGTGCCTTTTGTCATGCCCCTGC AGCTTCCTTCGTGTAACCC-3' complementary to bases 694-645 of the rat BDNF sequence (Maisonpierre et al., 1991; Kobayashi et al., 1996) which has been used previously by Ernfors et al. (1990). For the full-length trkB, we used a 45 mer oligonucleotide, 5'-GAGAGGGCTGGCAGAGTCATCGTCGTTGCTGATGACGGAAGCTGG-3' complementary to bases 1407-1363 of the rat trkB sequence (Middlemas et al., 1991; Kobayashi et al., 1996). This oligonucleotide is between the truncation site and the tyrosine kinase domain. These oligonucleotides were end-labeled with ^{35}S-ATP (NEN-Dupont; USA) using terminal deoxynucleotidyl transferase (Gibco BRL; USA) achieving a specific activity of 2.5 X 10^9 c.p.m./µg (Ausubel et al., 1987).

In each of 4 rats, fresh cryostat sections of spinal cords 8 hr, 2 d and 7 d after nerve repair with and without stimulation were removed from the freezer (– 80 C°). ISH of BDNF and trkB was carried out on 3 slides, the first, 7th and 13th of the total 8-20, and the 2nd, 8th and 14th slides, for each probe, respectively. Each slide contained sections at a distance of 450 µm along the longitudinal axis of the spinal cord. This provided sampling of motoneurons throughout the motoneuron pool along the 1500 µm longitudinal axis. *In situ* hybridization was carried out as described in detail by Verge et al. (1992). Briefly, spinal cord sections were hybridized to 10^6 c.p.m. of the probe in a l00 µl hybridization cocktail (deionised formamide, 20 x SSC, 50 x Denhardt's solution, 0.2 M sodium phosphate buffer, pH 7.0, dextran sulphate, 20 % sarcosyl). Control slides were hybridized as above in the presence of excess (400 X) unlabeled probe. After overnight hybridization at 42 C°, the slides were washed 4 times in 1 X SSC (0.15 M NaCl, 0.015 M sodium citrate, ph 7.0) for 15 min at 45 C°, rinsed briefly in a distilled water, and then dehydrated in ascending concentrations of ethanol to ensure that the sections adhere thereafter to the radiosensitive emulsion (Kodak NTP-2, diluted 1:1 in H_2O) during autoradiography. The sections were exposed to emulsion for adequate resolution of the silver grains activated by ^{35}S-ATP per probe in the dark for an 8 week period for BDNF mRNA and a 3 week period for trkB mRNA (Kobayashi et al., 1996). They were subsequently stained with neutral red, dehydrated, and embedded in permount.

3.2.5 Quantification of ISH signals

Six to 7 motoneurons per section (40 per slide; 30 motoneurons/rat) were randomly chosen for analysis in each of 4 rats. Neuronal cell bodies and nuclei were visualized and traced at 20-40 x under UV fluorescence using barrier filters of 430 nM for FG and 580 nM for FR, and the area fraction occupied by silver grains (i.e., grain density) was then automatically measured using Image-Pro Plus (Media

Cybernetics) from the corresponding dark-field image. Grain density, which represents mRNA per grain, was corrected by subtracting background autoradiographic signal. For each motoneuron, the fraction of the area occupied by autoradiographic silver grains was multiplied by the total cell area and expressed as a percentage of the signal. For each condition the mean percentage silver grain signal (±S.D.) was calculated. The investigator was unaware of the time point and which oligonucleotide probe had been applied to the slide.

3.2.6 Statistical analysis

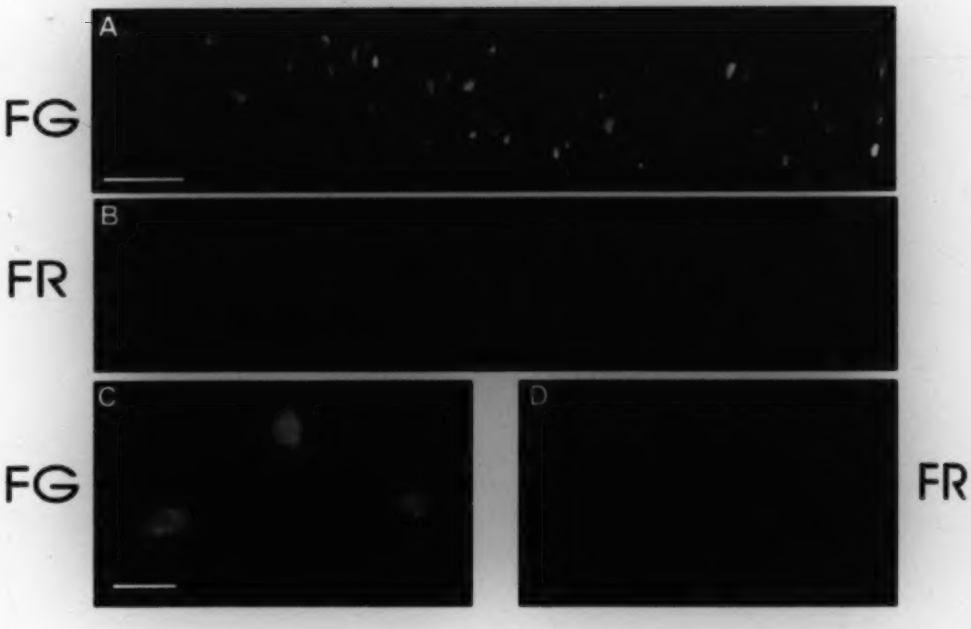
A one-way ANOVA was used to compare the mean percentage silver grain signal (±S.D.) of the axotomized and the contralateral control motoneurons. A multi-factorial ANOVA was used to compare the mean percentage silver grain signal (±S.D.) among all time points. The same comparison was used for the stimulation. Statistical significance was set at the 0.05 level.

3.3 RESULTS

3.3.1 Intramuscular injection of FG or FR is effective in labeling femoral motoneurons

We have previously labeled motoneurons by transecting the peripheral nerve and exposing the cut end to tracers [(Brushart et al., 1998; Al-Majed et al., 2000 (chapter 2)]. However, in the current experiments, cutting and prelabeling the cut nerve prior to nerve suture would impose either a preconditioning lesion (Brushart et al., 1998) if the labeling was carried out prior to the nerve surgery, or alter the nerve stump at the time of surgery if the surgical repair was carried out immediately. Moreover, since the retrograde transport of these dyes from the cut femoral nerve requires 3 days (Al-Majed et al., 2000) (chapter 2), the nerve application would obviate the ability to measure the early changes in gene expression in the cut and regenerating motoneurons. We thus backlabeled femoral motoneurons with flourescent tracers (FG or FR) by injection into the quadriceps muscle, 2 weeks prior to femoral nerve section and surgical repair. This method was very effective as shown in Fig. 3.1, where the dyes are seen both in the somata and dendrites of the motoneurons. The normal spatial distribution of backlabeled motoneurons in the lumbar spinal cord and the finding that the mean (±S.E) number (288±15) of motoneurons backlabeled via intramuscular injection almost reached the number of motoneurons which were labeled by exposing cut femoral nerve to dyes (338±6) (Al-Majed et al., 2000) (chapter 2), demonstrated the effectiveness of the method (see also Glover et al., 1986; Piehl et al., 1993; Richmond et al., 1994; Mohajeri et al., 1998; Cheng et al., 1998).

FIGURE 3.1. Effective backlabeling of femoral motoneurons in the lumbar sacral spinal cord by intramuscular injection of A) Fluorogold (FG) or B) fluororuby (FR) into the quadriceps muscles in both legs of an adult rat. **A**,**B**, *Low power* (*20 x*) fluorescence micrographs of the ventral horn in longitudinal sections from rat lumber spinal cord after intramuscular injection of *FG* (**A**) and *FR* (**B**) in the quadriceps muscle. Scale bar *200 μm*. **C**,**D**, *High-power* (*40 x*) magnification of motoneurons cell bodies and their dendrites effectively labeled with *FG* (**C**) and *FR* (**D**). Note the effective backlabeling of both soma and dendrites of femoral motoneurons. Scale bar *50 μm*


A
FG
B
FR
C
FG
D
FR

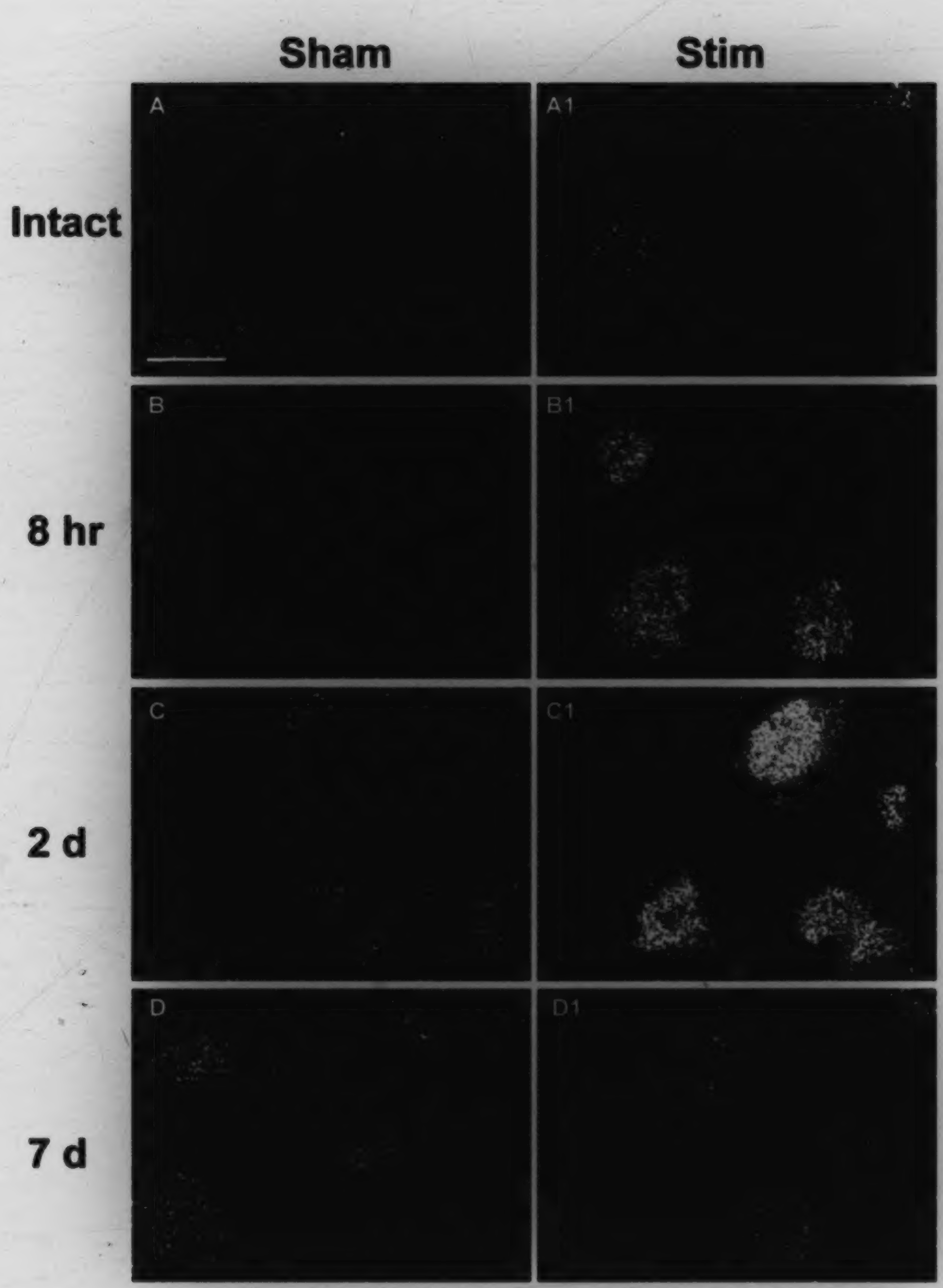
3.3.2 Expression of BDNF mRNA in stimulated and sham-stimulated motoneurons after femoral nerve cut and repair

ISH with 8 weeks exposure of spinal cord cross-sections to emulsion for autoradiography, was used to semi-quantify BDNF mRNA expression in regenerating motoneurons subjected to stimulation or sham-stimulation as well as in normal motoneurons on the contralateral side of the spinal cord. Control slides revealed no signal for BDNF in motoneurons. Low but detectable levels of BDNF mRNA expression were seen in the intact femoral motoneurons (Fig. 3.2A,A1). Eight hr and 2 d after femoral nerve transection and surgical repair with sham-stimulation, the signals were similar to those in the contralateral intact side (Fig. 3.2A-C). Signals were increased 7 d later (Fig. 3.2D). The representative motoneurons shown in Fig. 3.2 were identified as femoral motoneurons by retrograde labelling with FG or FR (not shown). Although there was some degradation, a sufficient amount remained for identification due to the stability of the retrograde dyes (Peihl et al., 1993; Cheng et al., 1998).

When repaired nerves were subjected to 1 hr stimulation at 20 Hz, silver grain densities increased dramatically and earlier, 8 hr and 2 d after axotomy and nerve repair (Fig. 3.2B1,C1). In fact, the very high density of silver grains, particularly at 2 d, clearly delineates the stimulated motoneurons from background staining, even without visualization of the retrograde labels. Moreover, the density of silver grains at both 8 hr and 2 d after nerve repair and stimulation was obviously higher than the unstimulated motoneurons even at 7 d (cf. Fig. 3.2B1,C1 and D).

By 7 d, the grain density in the stimulated regenerating motoneurons declined to levels seen at 7 d in the sham-stimulated regenerating motoneurons (cf. Fig. 3.2D, D1).

FIGURE 3.2. Dark field micrographs of ISH with ^{35}S-labeled oligonucleotide probe to detect BDNF mRNA in rat femoral motoneurons. Motoneurons were subjected either to *sham-stimulation* (**A-D**) or to *1 hr 20 Hz electrical stimulation* (**A1-D1**). **A**, **A1**. Contralateral (*intact*) femoral motoneurons. **B1-D1**. Femoral motoneurons, *8 hr* (**B, B1**), *2 d* (**C, C1**) and *7 d* (**D, D1**) after nerve cut and repair. Note the low level expression of BDNF mRNA in intact motoneurons and the delayed (*7 d*) increase in grain density in sham-stimulated femoral motoneurons (A, A1, B-D) as compared to the earlier (*8 hr and 2 d*) increases seen in stimulated regenerating motoneurons (B1-D1). Scale bar *50 μm*


Sham
Stim
Intact
8 hr
2 d
7 d
A
A1
B
B1
C
C1
D
D1

Quantification of the autoradiographic silver grains (signal/cell) revealed limited variability in the BDNF ISH signal (BDNF mRNA levels) in intact motoneurons sampled from unoperated side of all rats (Fig. 3.3A). The BDNF ISH signal/cell showed the same normal distribution 8 hr and 2 d after femoral nerve repair and sham-stimulation and was not statistically different from the contralateral intact side ($P > 0.05$, Fig. 3.3B,C). Mean (± SE) values for the cellular area occupied by silver grain were 10.2 ± 0.2 and 9.7 ± 0.2 for intact controls for sham and stimulated groups, respectively, and 9.8 ± 0.2 and 9.9 ± 0.2 for 8 hr and 2 d after nerve repair and sham-stimulation, respectively. The BDNF ISH signals increased to significantly higher levels by 7 d (20.1 ± 0.2, $P < 0.01$) but the frequency distribution was unchanged (Fig. 3.3D). In contrast to unchanged levels of mRNA at 8 hr and 2 d with sham-stimulation relative to contralateral intact motoneurons, there was a dramatic increase in the mean values of the BDNF ISH signals in the stimulated femoral motoneurons after nerve section and repair (34.0 ± 3.0 for 8 hr and 61.8 ± 5.0 for 2 d, $P < 0.01$). As shown in Fig. 3.3, the histograms for the BDNF ISH signals in the stimulated motoneurons were shifted far to the right of the signals in the sham stimulated motoneurons, 8 hr and 2 d after nerve repair and stimulation. Note that the distributions were widened for the stimulated motoneurons such that the standard deviations around the mean values shown in Fig. 3.4 were obviously larger than in the sham-stimulated motoneurons. As reported in axotomized sciatic and facial motoneurons (Funakoshi et al., 1993; Kobayashi et al., 1996), the upregulation of BDNF in regenerating and stimulated femoral motoneurons was transient, with high levels of BDNF mRNA seen at 2 d, receding by 7 d (Fig. 3.3D). At 7 d, BDNF mRNA levels were the same as the sham-stimulated axotomized and surgically repaired motoneurons (Fig. 3.3). The remarkable consistency in the data among animals is seen in Fig. 3.4 where the mean (± SD) for at least 30 motoneurons is shown for 4 animals at each time point in the stimulated and sham-stimulated groups. The BDNF ISH signal increased 6-fold by 2 d after electrical stimulation, in contrast to slightly less than a 2-fold increase by 7 d in the sham-stimulated group.

FIGURE 3.3. Frequency histograms of BDNF ISH signal/cell profile (mRNA expression per motoneuron profile) in regenerating femoral motoneurons subjected to *sham-stimulation* (*open histograms*) or to *1 hr* continuous *20 Hz frequency electrical stimulation* (*stim, filled histograms*). **A**, Contralateral (*intact*) femoral motoneurons and *regenerating motoneurons 8 hr* (**B**), *2 d* (**C**) and *7 d* (**D**) after femoral nerve cut and surgical repair. Note that intact motoneurons express low levels of BDNF mRNA which remain at the same level, 8 hr and 2 d after axotomy and nerve repair, until the levels are doubled at 7 days in sham stimulated regenerating motoneurons (*open bars*). This contrasts with an accelerated and greatly enhanced BDNF expression 8 hr and 2 d after electrical stimulation (*filled bars*). Electrical stimulation elevates the mean mRNA expression in regenerating motoneurons as well as increases the range.

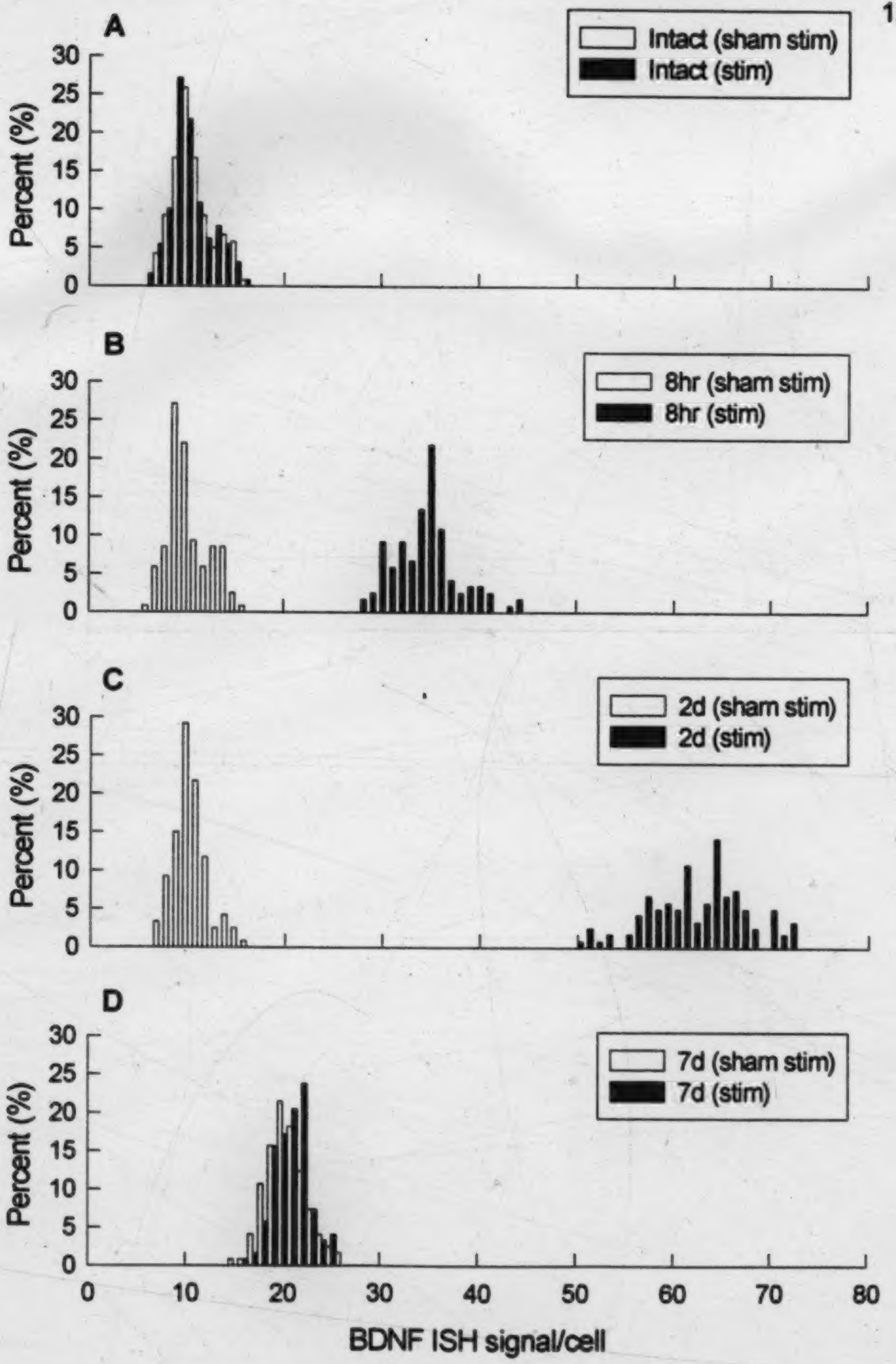
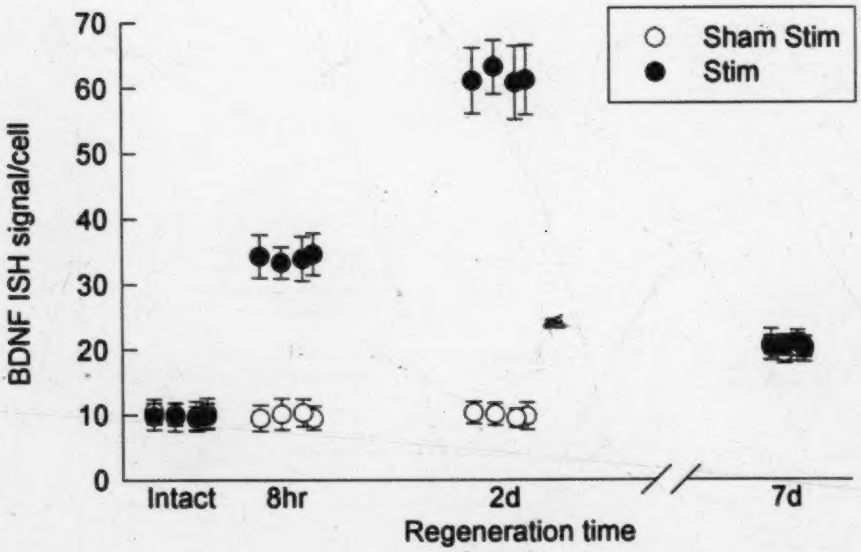

A
Intact (sham stim)
Intact (stim)
B
8hr (sham stim)
8hr (stim)
C
2d (sham stim)
2d (stim)
D
7d (sham stim)
7d (stim)
Percent (%)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
BDNF ISH signal/cell

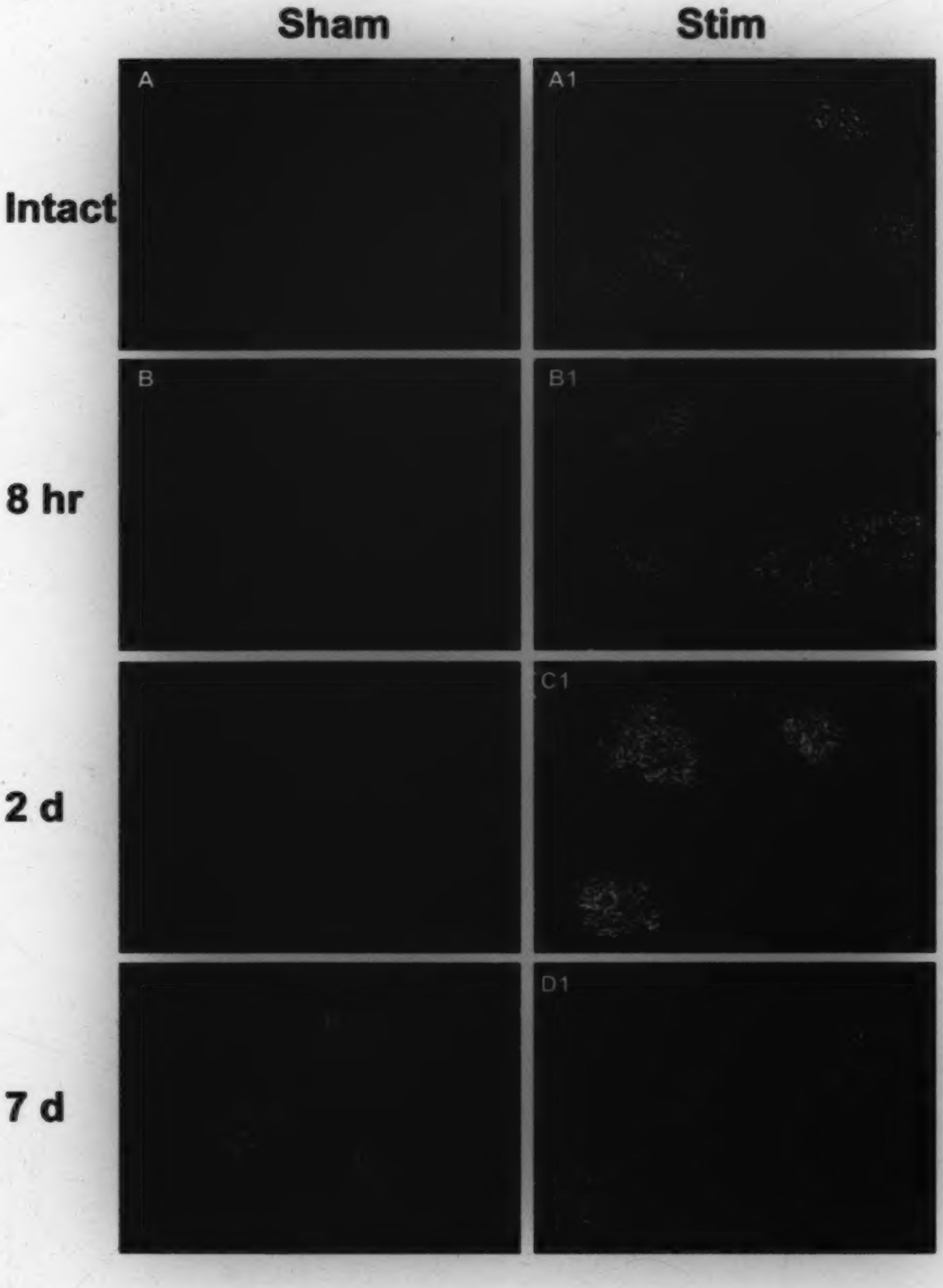
FIGURE 3.4. The acceleration and elevation of motoneuron expression of BDNF mRNA in response to 1 hr 20 Hz electrical stimulation of axotomized and surgically repaired femoral motoneurons. The mean ± SD of the BDNF ISH signals/cell is plotted for motoneurons subjected to *sham-stimulation* (*open symbols*) and *1 hr 20 Hz electrical stimulation* (*filled symbols*) vs. survival time (*8 hr, 2 d* and *7 d*) after femoral nerve and repair. Note that the mean ± SD of the values obtained for 4 different rats per time point are displayed along the time-axis for clarity. The mean values of ISH signals/cell profile in individual rats for each time point were remarkably similar in both sham-stimulated and stimulated motoneurons. Backfilling of motoneurons alternatively with either FG or FR in the group of 4 rats per time point, demonstrates that the retrograde labeling with either dye did not affect mRNA expression. Note also the increase in variability around the mean values for the stimulated motoneurons as shown by the larger SDs.


BDNF ISH signal/cell
70
60
50
40
30
20
10
0
Intact
8hr
2d
7d
Regeneration time
○ Sham Stim
● Stim

3.3.3 Expression of trkB mRNA in stimulated and sham-stimulated motoneurons after femoral nerve cut and repair

The hypothesis that stimulation induced acceleration of motor axonal regeneration is linked to accelerated and more pronounced upregulation of BDNF, predicts that stimulation will similarly accelerate and upregulate the expression of the high-affinity receptor for BDNF in the same motoneurons. We used an oligonucleotide probe which specifically recognises the full-length trkB receptor including the tyrosine kinase domain (Middlemas et al., 1991; Kobayashi et al., 1996) to investigate the relative change in expression of trkB receptors in sham-stimulated and 20 Hz stimulated motoneurons, 8 hr, 2 d and 7 d after femoral nerve cut and repair. ISH with 3 weeks exposure of spinal cord cross-sections to emulsion for autoradiography, was used to semi-quantify trkB mRNA expression. Control slides revealed no signal for trkB in motoneurons. As demonstrated for BDNF mRNA, there was a delayed upregulation of trkB mRNA in sham-stimulated motoneurons in contrast to the rapid upregulation by 8 hr and 2 d after 1 hr stimulation (Fig. 3.5B-C, B1-C1). By 7 d after nerve repair, the expression of trkB was similar in sham-stimulated and 20 Hz stimulated motoneurons (Fig. 3.5D,D1).

FIGURE 3.5. Dark field micrographs of ISH with ^{35}S labeled oligonucleotide probe to detect the full-length trkB mRNA in rat intact and regenerating femoral motoneurons which are subjected to *sham-stimulation* (**A-D**) or to *1 hr 20 Hz electrical stimulation* (**A1-D1**). **A**, **A1**, Contralateral (*intact*) femoral motoneurons. **B1-D1**, Femoral motoneurons *8 hr* (**B, B1**), *2 d* (**C, C1**) and *7 d* (**D, D1**) after nerve cut and repair. Note there is a delay in upregulation of trkB in the sham-stimulated regenerating motoneurons until 7 d after surgical repair similar to that found for BDNF as shown in Figure 2. Note also the earlier and obviously greater elevation of trkB signals after electrical stimulation of the regenerating motoneurons, as found for BDNF in the same motoneurons. However, the trkB ISH signal per cell profile appeared to be less for trkB than BDNF at 2 d (cf C1 in Fig. 3.2). Scale bar *50 μm*


Sham
Stim
Intact
8 hr
2 d
7 d
A
A1
B
B1
C1
D1

The frequency distributions of trkB ISH signals/cell in the axotomized and surgically repaired motoneurons are compared for the sham stimulated and 1 hr stimulated regenerating motoneurons in Fig. 3.6. Interestingly, the effect of 1 hr 20 Hz electrical stimulation in accelerating the upregulation of trkB mRNA, like BDNF mRNA, was accompanied by the same broadening of the distribution of the trkB ISH signals/cell. However, electrical stimulation amplified trkB mRNA expression to a lesser extent than BDNF mRNA expression. This is seen as a smaller rightward shift of the histograms along the trkB ISH signal axis at 8 hr and 2 d and a 4.5 fold increase in the mean values by 2 d in contrast to the 6-fold increase in BDNF mRNA expression at 2 d (cf. Figs. 3.7 and 3.4). However, the 2-fold elevation of trkB mRNA signals at 7 d for the stimulated and sham-stimulated motoneurons (Fig. 3.7) was the same as the 2-fold increase seen for BDNF mRNA at the same time after femoral nerve section and nerve repair (Fig. 3.4). These data, showing a parallel acceleration and elevation in expression of the neurotrophin BDNF and its high affinity receptor trkB in motoneurons, support the hypothesis that BDNF plays a central role in the acceleration of motor axonal regeneration by short-term electrical stimulation.

FIGURE 3.6. Frequency histograms of full-length trkB ISH signal/cell profile of intact femoral motoneurons and axotomized and surgically repaired femoral nerves subjected to *sham-stimulation* (*open histograms*) and to *1 hr 20 Hz electrical stimulation* immediately after surgical repair (*filled histograms*). **A**, Contralateral (*intact*) femoral motoneurons, and *regenerating motoneurons 8 hr* (**B**), *2 d* (**C**) and *7 d* (**D**)after nerve cut and surgical repair. Note that electrical stimulation of axotomized and surgically repaired femoral motoneurons accelerates and enhances mRNA expression of the full length trkB receptor as for the endogenous ligand BDNF shown in Fig. 4.3. However, the elevation in trkB mRNA is relatively less than for BDNF (note the same scales on the X-axis in both figures).

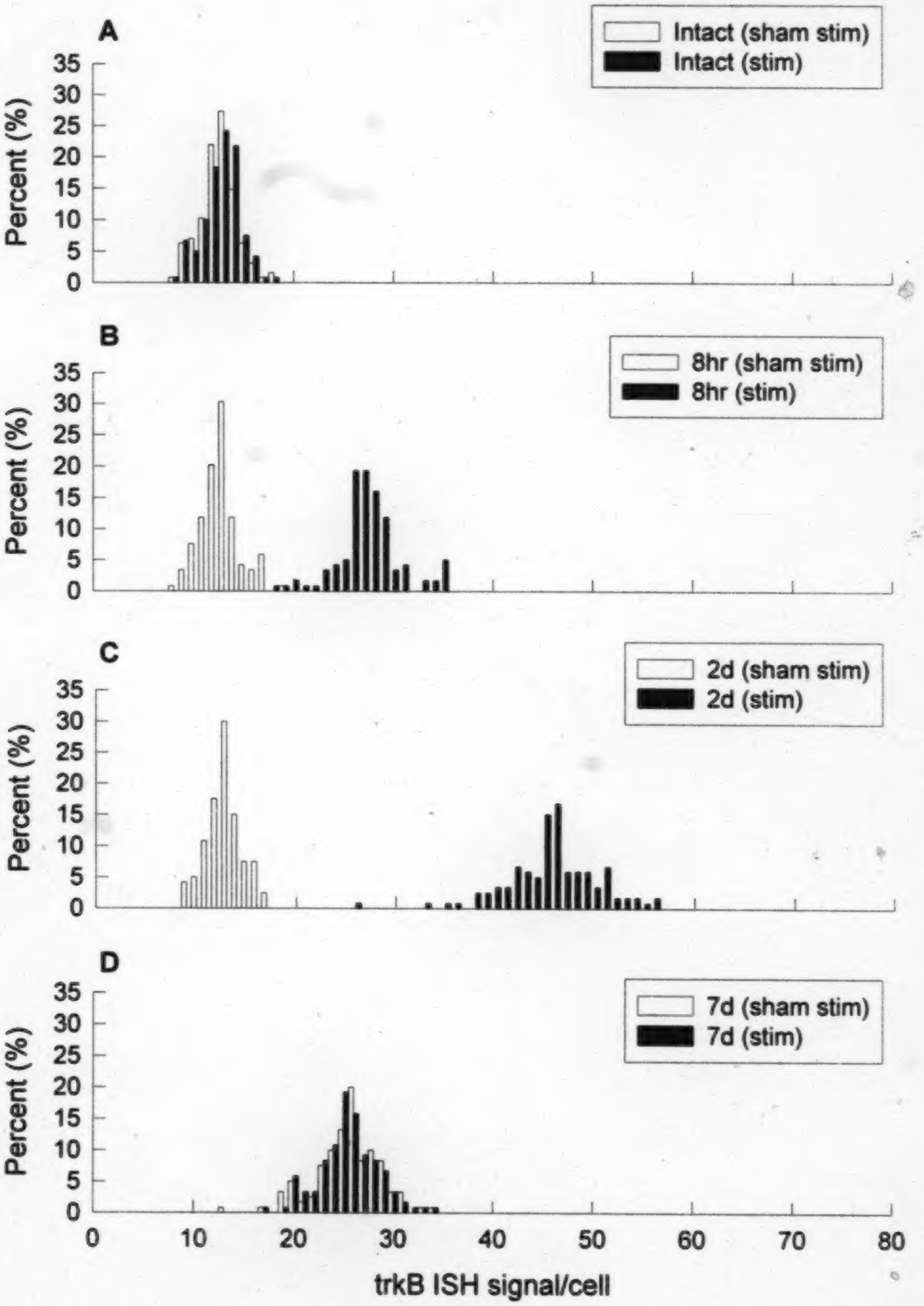
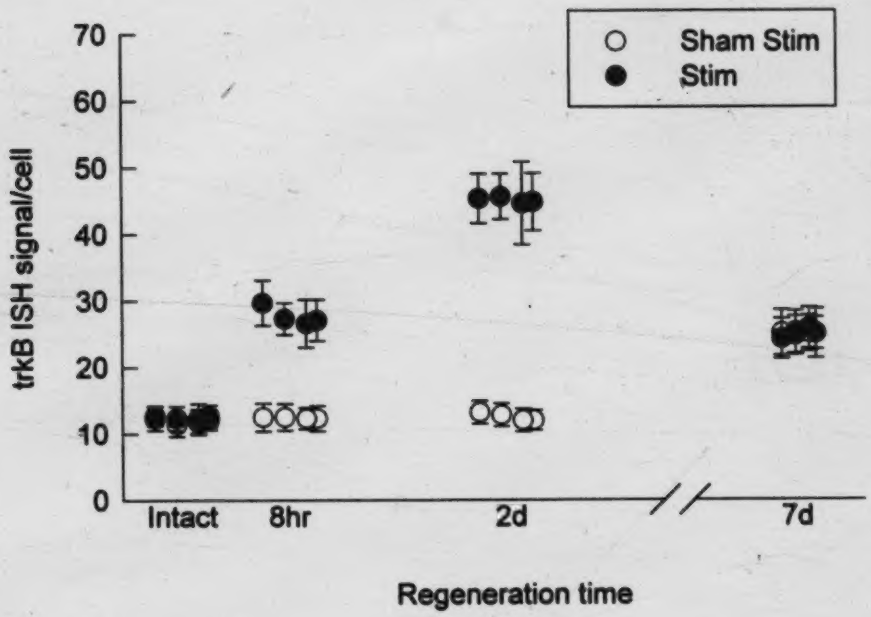

A
Intact (sham stim)
Intact (stim)
B
8hr (sham stim)
8hr (stim)
C
2d (sham stim)
2d (stim)
D
7d (sham stim)
7d (stim)
Percent (%)
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
70
80
trkB ISH signal/cell

FIGURE 3.7. The mean ± SD of the ISH signal/cell profile (mRNA expression) detecting full-length trkB mRNA in *intact* and *regenerating femoral motoneurons, 8 hr, 2 d* and *7 d* after *1 hr sham-stimulation* (*open symbols*) and *20 Hz stimulation* (*filled symbols*). The mean ± SD values are separated at each time point on the X-axis for comparison between data obtained from individual rats. Electrical stimulation of axotomized motoneurons upregulated mRNA expression of trkB earlier and more dramatically than sham-stimulation. Note the relatively smaller elevation of trkB expression as compared to BDNF expression in Fig. 3.4.


Sham Stim
Stim
trkB ISH signal/cell
70
60
50
40
30
20
10
0
Intact
8hr
2d
7d
Regeneration time

3.4 DISCUSSION

In this study, we used semi-quantitative ISH to demonstrate that short-term (1 hr) continuous 20 Hz stimulation greatly accelerates expression of the genes for BDNF and its high-affinity trkB receptor in regenerating motoneurons. These findings support the hypothesis that acceleration of BDNF upregulation by electrical stimulation of axotomized motoneurons plays a key role in accelerating outgrowth of their axons, which we demonstrated previously (Al-Majed et al., 2000) (Chapter 2).

3.4.1 Upregulation of BDNF and trkB mRNAs and axonal regeneration

BDNF and trkB mRNA were detected at low levels in intact rat femoral motoneurons and were increased after axotomy, consistent with previous findings in rat sciatic and facial motoneurons (Funakoshi et al., 1993, Koliatsos et al., 1994, Piehl et al., 1994, Kobayashi et al., 1996). Piehl et al. (1994), using ISH, reported a 2-fold increase in full-length trkB mRNA in sciatic motoneurons 3 d after axotomy. Kobayashi et al. (1996) confirmed these findings in axotomized facial motoneurons, reporting the same increase in trkB mRNA 2 d after axotomy. They also found a transient 4-fold increases in BDNF mRNA which started to decline 4 d after injury. We demonstrate here that BDNF and trkB mRNA expression are also upregulated by axotomy under conditions when axonal regeneration is encouraged by surgical repair. However our data indicate that femoral motoneurons do not increase BDNF mRNA expression until one week after femoral nerve cut and surgical repair (Fig.

3.2D, 3.3D, 3.4) in contrast to the relatively shorter periods when regeneration does not occur after axotomy.

Understanding the function of this increase in BDNF expression by the axotomized motoneurons requires an understanding of the expression of the trkB receptor for BDNF in the motoneurons. The upregulation of BDNF by regenerating motoneurons is associated with the same (2-fold) increase in its receptor (trkB) mRNA expression (Fig. 3.5D, 3.6D, 3.7) as reported by Piehl et al. (1994), 3 days after axotomy. This suggests that BDNF acts in an autocrine fashion providing trophic support after axotomy and target deprivation. The increase in trkB expression in axotomized motoneurons may enhance their responsiveness to BDNF. In addition, BDNF has been shown to prevent the death of axotomized adult rat sciatic and facial motoneurons (Yan et al., 1992; Sendtner et al., 1992) and to rescue developing chick motoneurons in vivo from naturally occurring cell death (Oppenheim et al., 1992). Thus, a role of BDNF in axonal regeneration has been implicated, however, it has not been certainly demonstrated until recently (Boyd and Gordon, 2000). Several studies have demonstrated that local application of BDNF can enhance regenerative sprouting in CNS axons (e.g. Tuszynski et al., 1996; Xu et al., 1995; Ye and Houle, 1997). Infusion of recombinant human BDNF into the vicinity of axotomized rubrospinal neurons (RSNs), between days 7 and 14 after axotomy was found to increase the number of axotomized RSNs that regenerated into a peripheral nerve graft concurrent with upregulation of regeneration-associated genes Tα1-tubulin and GAP-43 (Kobayashi et al., 1997). In this study, we used the femoral nerve transection and surgical repair model. Motoneurons regenerate over a protracted period of up to 10 weeks as they progressively reinnervate appropriate muscle pathways (Al-Majed et al., 2000) (chapter 2). The relative delay in the upregulation of BDNF and trkB in the femoral nerve model could thus contribute to the prolonged time required for all motoneurons to regenerate their axons over a 25 mm distance (Al-Majed et al., 2000) (chapter 2).

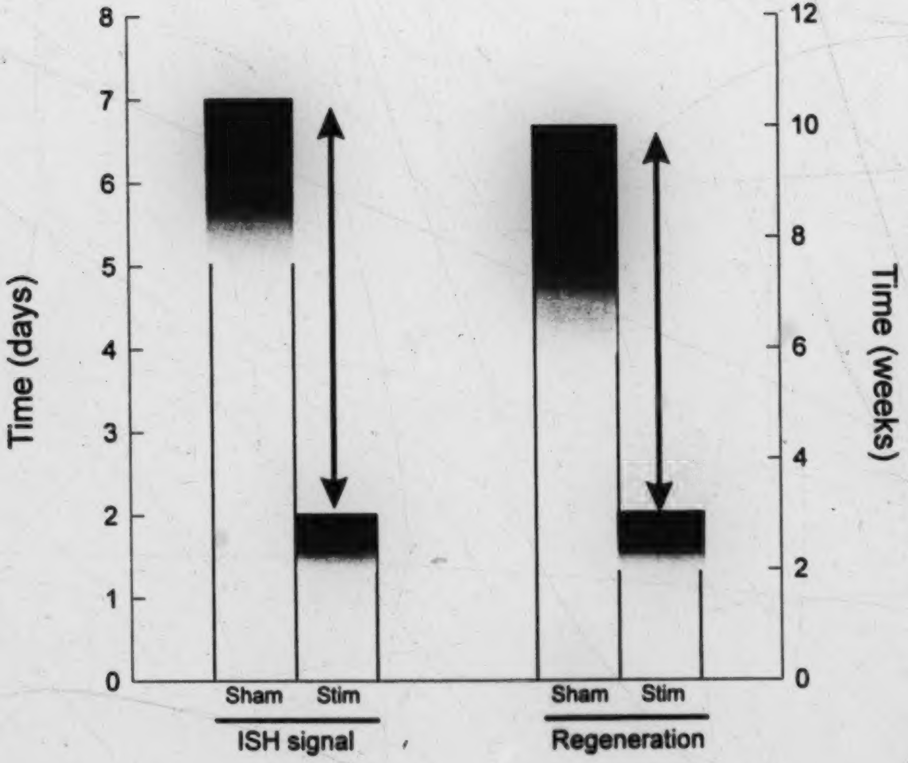
3.4.2 Effects of 1 hr electrical stimulation on motoneuron expression of BDNF and trkB

We show here that 1 hr 20 Hz electrical stimulation of the proximal stump of the axotomized and repaired femoral nerve, dramatically increased and accelerated motoneuron expression of BDNF and trkB mRNA in association with the dramatic acceleration of motor axonal regeneration demonstrated previously (Al-Majed et al., 2000) (Chapter 2). The strong temporal association of upregulation of BDNF and trkB in regenerating motoneurons and the accelerated motor axonal regeneration in response to 1 hr stimulation provides strong support for a central role of BDNF in the strong accelerating influence of stimulation on motor axonal regeneration. While several studies have shown that the expression of BDNF and, to a lesser extent, trkB mRNA can be induced by neuronal activity, we demonstrate a direct association between acceleration of motor axonal regeneration and upregulation of BDNF and trkB receptors in electrically stimulated motoneurons. *In vivo* and *in vitro* studies have shown that BDNF mRNA in central neurons can be induced by a wide variety of depolarizing events such as potassium depolarization, kindling, spreading depression, application of kainic acid and cholinergic agonists, as well as GABA-A antagonists (Zafra et al., 1990,1991; Ballatin et al., 1991; Ernfors et al., 1991; Kokaia et al., 1993; Wetmore et al., 1994). In addition trkB mRNA expression is upregulated by kainic acid-induced seizures in the cortex (Aloyz et al., 1999). Several recent studies have shown that BDNF and trkB mRNAs levels are also induced by physiological stimuli. For example, visual experience regulates the expression of BDNF in both the newborn and adult rat visual system (Castrén et al., 1992). These BDNF and trkB mRNAs increases occur rapidly, as early as 1 hr and 3 hr after neuronal activity.

Low frequency electrical stimulation applied immediately after surgical repair of the cut femoral nerve dramatically accelerates axonal regeneration and PMR (Al-Majed et al., 2000) (Chapter 2). The rapid induction of BDNF and trkB expression in femoral motoneurons 8 hr and 2 d after 1 hr stimulation of the cut and surgically repaired femoral nerves (Fig. 3.4 and 3.7) might account for the dramatic enhancement of motor axonal regeneration. Normally 200 motoneurons regenerate their axons 25 mm from the suture site by 2 to 3 weeks. This number progressively doubles over an experimental time course by 8 to 10 weeks (Fig. 3.3 in Al-Majed et al., 2000) (chapter 2). At 3 mm/d regeneration rate, all motoneurons which are expected to regenerate their axons over 25 mm (to the point of application of retrograde dyes) in 2 to 3 weeks did not do so until 8 to 10 weeks had elapsed. This long period of 8 to 10 weeks is consistent with long and variable delays for regenerating axons to successfully cross the surgical gap and enter the distal nerve stumps (Al-Majed et al., 2000) (chapter 2). However the dramatic acceleration of axonal regeneration by 1 hr stimulation and the regeneration of almost all motoneurons by 3 weeks provides strong evidence for a dramatic acceleration of axonal outgrowth.

The very pronounced and accelerated upregulation of BDNF and trkB by 8 hr and 2 d by electrical stimulation is directly associated with the dramatic acceleration in number of motoneurons which regenerate over 25 mm distance by 2 to 3 weeks. As illustrated in Fig. 3.8, the upregulation of BDNF and trkB in electrically stimulated regenerating motoneurons is accelerated by 3.5 times. This is the same as the acceleration (3.3 times) of the regeneration of all motoneurons from 8-10 weeks to 2-3 weeks by 1 hr electrical stimulation. This strong correspondence between the time course of upregulation of BDNF and trkB and acceleration of motor axonal regeneration after 1 hr stimulation provides strong support for a direct link between BDNF expression in motoneurons and axonal outgrowth.

FIGURE 3.8. The relative time point in days that BDNF and trkB expression were maximally elevated in regenerating femoral motoneurons after *1 hr sham-stimulation* or *20 Hz electrical stimulation* is compared with the *relative time in weeks* that all axotomized motoneurons regenerated over a distance of *25 mm*. The effect of the electrical stimulation was to accelerate expression of BDNF and trkB mRNA and the staggered motor axonal regeneration of axotomized motoneurons by the same factor of **3.3** to **3.5** fold. *Progressive shading* from dark to no shading is used in the bar graphs to better represent the range in time for parameters which were measured at fixed time points.


Time (days)
8
7
6
5
4
3
2
1
0
Time (weeks)
12
10
8
6
4
2
0
Sham
Stim
ISH signal
Sham
Stim
Regeneration

The mechanism of BDNF and trkB induction by electrical stimulation is unknown. It has been established that the upregulation of BDNF is associated with membrane depolarization induced by elevated extracellular potassium concentrations (50 m*M*) (Zafra et al., 1990). This demonstrated effect of potassium induced depolarization depends on extracellular Ca^{2+} and is blocked by the L-type Ca^{2+} channel blocker, nifedipine. Conversely, Ca^{2+} ionophores and activators of L-type calcium channels mimicked the effect of potassium depolarization in increasing BDNF gene expression. The ability of calmodulin inhibitors to block the activity-dependent increase in BDNF mRNA suggests that these effects are mediated by Ca^{2+}-calmodulin-dependent protein kinases (CaM kinase) (Zafra et al., 1992). Moreover, recent reports have indicated that Ca^{2+} influx triggers phosphorylation of cAMP response element binding (CREB) at ser-133 via CaM kinase IV which, by binding to a critical Ca^{2+} response element (CRE) within the BDNF gene, activates BDNF transcription (Shieh et al., 1998; Tao et al., 1998). In addition to CaM kinase IV, other kinases may also contribute to Ca^{2+} regulation of CREB phosphorylation and BDNF transcription. Likely possibilities are members of the pp90rsk family (RSK 1-3) and the MAP kinase-activated protein (MAPKAP) kinases 1-2 that have been recently shown to mediate growth factor-induced CREB ser-133 phosphorylation (Xing et al., 1998). The RSKs and MAPKAP kinases are both activated by the ras signaling pathway, and ras is activated by Ca^{2+} influx through L-type Ca^{2+} channels (Rose et al., 1994; Xing et al., 1996).

3.4.3 Significance

One hr of electrical stimulation, which has been shown to promote motor axonal regeneration and PMR, dramatically increased and accelerated motoneuron expression of BDNF and its receptor trkB after femoral nerve cut and surgical repair. It is likely that this BDNF acts on the motoneurons in an autocrine and/or paracrine fashion to promote motor axonal regeneration. An understanding of the mechanism(s) by which electrical stimulation mediates its positive effects on peripheral nerve regeneration will provide new insights into the molecular basis of regeneration. This might allow us to extend the experimental findings to clinical usage, possibly in association with pharmacological tools such as those which have been shown to increase BDNF mRNA in central neurons (Zafra et al., 1990,1991; Ballatin et al., 1991; Ernfors et al., 1991). These could be developed to improve recovery after injuries to peripheral nerve trunks containing both cutaneous and muscle axons.

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CHAPTER 4

4.0 REDUCTION OF NEUROFILAMENT AND INDUCTION OF TUBULIN AND GAP-43 GENES EXPRESSION IN MOTONEURONS BY ELECTRICAL STIMULATION: A POSSIBLE MECHANISM FOR ELECTRICAL STIMULATION EFFECT ON MOTOR AXONAL REGENERATION

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4.1 INTRODUCTION

Axonal injuries leading to an interruption of the contact between a motoneuron and its muscle fibers have profound effects on the parent cell body (Lieberman, 1971; Kreutzberg, 1982; Aldskogius and Svensson, 1993). Apart from morphological changes occurring in lesioned motoneurons, the axotomy response includes a major shift in the gene expression, such as the prominent upregulation of growth associated genes, most prominently GAP-43 (for review, see Skene, 1989; Benowitz and Routtenberg, 1997; Fu and Gordon, 1997), immediate early genes such as c-jun (for review, see Herdegen et al., 1997), and cytoskeletal proteins such as tubulin (for review, see Bisby and Tetzlaff, 1992). These changes occur concomitant to the downregulation of neurofilament (for review, see Bisby and Tetzlaff, 1992) and neurotransmitter enzymes (Kou et al., 1995; Fernandes et al., 1998). Chronically axotomized motoneurons also failed to sustain expression of Tα1-tubulin and GAP-43 which indicated that the poor regenerative capacity of chronically axotomized motoneurons is due to failure of the motoneurons to sustain expression of these genes (Petrov et al., 1996).

During axonal regeneration in the adult, axonal outgrowth rates correlate with the axonal transport of tubulin in slow component b (SCb) (Wujek et al., 1983). Upregulation of tubulin concurrent with rapid downregulation of neurofilament gene expression has been suggested to decrease neurofilament/tubulin ratios, allowing more rapid transport of tubulin and faster axonal elongation (Hoffman and Lasek, 1980; Hoffman et al., 1985). The expression of GAP-43 is strongly correlated to events involving growth or regeneration (Skene and Willard 1981; Tetzlaff et al., 1989; Caroni and Becker, 1992). Functionally, increased gene expression of the GAP-43 family has been shown to increase the spontaneous sprouting of neurons *in vivo* (Aigner et al., 1995) and to prevent growth cone collapse in response to myelin *in vitro* (Aigner and Caroni, 1995; for review, see Caroni, 1997).

Motor axons regenerate more rapidly after nerve transection and suture of peripheral nerve if they received 1 hr 20 Hz continuous electrical stimulation (Al-Majed et al., 2000a) (Chapter 2). Stimulation has been also found to accelerate and increase expression of the genes for brain-derived neurotrophic factor (BDNF) and its high affinity trkB receptor in regenerating motoneurons after femoral nerve cut and suture (Al-Majed et al., 2000b) (Chapter 3). An understanding of the mechanism(s) by which stimulation promotes axonal regeneration should provide important information about the critical factors which regulate peripheral nerve regeneration. In the present study we have focussed on the role of changes in the cell body in the positive effect of stimulation on axonal regeneration. In particular, we were interested in whether stimulation accelerates the changes in the gene expression of the major cytoskeletal proteins, neurofilament and tubulin as well as the growth associated protein, GAP-43.

A conditioning lesion which accelerates axonal regeneration (McQuarrie, 1985) reduces the motoneurons's synthesis of neurofilament (Tetzlaff et al., 1996). Several studies have shown that neuronal activity can regulate gene expression of cytoskeletal proteins and GAP-43. For example, brief potassium depolarization has been demonstrated to decrease levels of neurofilament protein in CNS cultures (Bar et al., 1993; Whitson et Al., 1995a; Whitson et Al., 1995b; Kampfl et al., 1996). Stimuli that evoke long-term potentiation (LTP) *in vivo* (Namgung et al., 1997) induce GAP-43 gene expression in hippocampal neurons. Electrical stimulation might alter gene expression of these proteins indirectly via BDNF. BDNF was found to increase the number of axotomized RSNs that regenerated into a peripheral nerve graft concurrent with upregulation of Tα1-tubulin and GAP-43 mRNA (Kobayashi et al., 1997). Moreover BDNF has been shown to increase the intraretinal branch length of injured retinal ganglion cells of adult rats concurrent with upregulation of GAP-43 mRNA (Fournier et al., 1997)

The present experiments were designed to assess the importance of motoneuronal cell body responses, specifically, expression of regeneration associated genes (RAGs) and neurofilament genes for promoting axonal regeneration by electrical stimulation. Our specific objective was to semi-quantitative medium molecular weight neurofilament protein (NFM), Tα1-tubulin and GAP-43 gene expression using *in situ* hybridization (ISH) after femoral nerve cut, suture and 1 hr electrical stimulation.

4.2 MATERIALS AND METHODS

Twenty-four young adult (220-240 gm) female Sprague Dawley rats were used for the experiments. All surgical procedures were performed under aseptic conditions on animals deeply anesthetized with somnotol (30 mg/kg, i.p.). Experiments were approved by the local ethics committee (Health Science Laboratory Animal Services) under the guidelines of the Canadian Council for Animal Care.

4.2.1 Femoral nerve model

Experiments were performed on the adult rat femoral nerve in which motor axons regenerate in a staggered manner and preferentially reinnervate muscle nerve pathways (Al-Majed et al, 2000a) (Chapter 2). The femoral nerve normally contains cutaneous sensory fibers that innervate the skin via the saphenous nerve. These are intermingled with sensory and motor fibers destined for the quadriceps muscle via the quadriceps muscle nerve (Fig.1a in Al-Majed et al., 2000a) (Chapter 2). One third of the axons derive from α-motoneurons, which innervate the skeletal muscle fibers (Brushart & Seiler, 1987).

4.2.2 Nerve suture

Two weeks prior to femoral nerve transection and suture, Fuorogold (FG, Fluorochrome Inc. Denver, CO) or fluororuby (FR, dextran tetramethylrhodamine, Molecular. Probes, D-1817, Eugene, OR) was injected into quadriceps muscles of the left and right hindlimb in order to label the femoral motoneuron pools as previously described (Al-Majed et al., 2000b) (Chapter 3). In brief, a small skin incision of less than 1 mm in length was made over the belly of the quadriceps muscle for microinjection of the tracers, FG (7% in DMSO-saline) or FR (10% in DMSO-saline) into the belly of the quadriceps muscle (Richmond et al., 1994; Al-Majed et al., 2000b) (Chapter 3). Approximately 18 μl of the tracer was delivered over the course of 5 minutes to each muscle using a 26-gauge needle connected to a Hamilton microsyringe. The needle was advanced across the tendinous midsection separating the medial and lateral compartments and withdrawn slowly during the injection to leave a track of injected material across the 2 compartments close to its innervation band (Richmond et al. 1994; Al-Majed et al., 2000b) (Chapter 3). After careful retraction of the syringe to avoid leakage of tracer, the injection site was cleaned and the skin sutured.

Femoral nerve transection and suture was performed 2 weeks after injection to avoid lesion effects on motoneuron gene expression. The left proximal femoral nerve was sharply cut 20 mm proximal its bifurcation into cutaneous and muscle nerves. The proximal and distal stumps were then carefully aligned and surgically joined within a 4 mm long silastic nerve cuff (Dow Corning, 0.76 mm inner diameter) by placing a single stitch of 9-0 Ethicon (Ethicon) through the epineurium of the proximal and distal stumps under 40-power magnification, as described in detail by Al-Majed et al (2000a) (Chapter 3). Electrodes were placed proximal to the suture site immediately after nerve suture for supramaximal pulses (100 μs, 3V) for 1 hr, delivered in a continuous 20 Hz train by the implantable stimulator (experimental group; n=12) or sham stimulation with the stimulator off (n=12). During the 1 hr period, the injury site was kept moist by covering the exposed tissues with a saline moistened gauze. Subsequently the electrodes were removed, the wound was closed and the skin was sutured. Groups of 4 stimulated and 4 sham-stimulated rats were then allowed to survive for 8 hr, 2d, or 7d after nerve transection and suture. Thereafter, rats were then killed by an overdose of somnotol and the lumbar spinal cord (T11-L1) was removed immediately, fresh frozen in isopentane cooled to – 70 C°, and stored at – 80 C° until further processing to prevent RNA breakdown by RNases.

4.2.3 *In situ* hybridization

The fresh-frozen lumbar spinal cords (~1500 μm in length) were sectioned in the coronal plane at 12 μm on a freezing microtome (Jung CM 3000). Each spinal cord tissue section contained both left (axotomized) and right (contralateral control) motoneurons. Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) were used to collect 6 serial sections. The process was repeated 6 times on each slide, so that neighbouring sections on each slide were 72 μm apart, eliminating the possibilities of repetitive sampling of motoneurons. Eighteen-20 slides per animal were collected. Slides were stored at – 80 C°.

ISH was carried out under RNase free conditions. Synthetic oligonucleotide probes were used for ISH as described by Verge et al. (1992). In brief, we used a

50 mer oligonucleotide 5'-CCCAGTGATGCTTCCTGAAAATGTGCTAAATCTGGTCTCTTCACCCTCC-3' complementary to NFM mRNA (Julien et al., 1986; Tetzlaff et al., 1991; Fernandes et al, 1999), a 50 mer oligonucleotide 5'-AAACCCATCAGTGAAGTGGACGGCTCGGGTCTCTGACAAATCATTCA-3' complementary to the 3'-untranslated sequence of Tα1-tubulin mRNA (Kobayashi et al., 1997; Fernandes et al., 1999), and for GAP-43 a 50 mer oligonucleotide 5'-GCATCGGTAGTAGCAGAGCCATCTCCCTCCTTCTTC TCCACACCATCAGCAA-3' complementary to bases 220-270 (Basi et al., 1987; Kobayashi et al. 1997; Fernandes et al. 1999). These oligonucleotides were end-labeled with ^{35}S-ATP (NEN-Dupont, USA) using terminal deoxynucleotidyl transferase (Gibco BRL, USA) achieving a specific activity of 2.5×10^9 c.p.m./µg (Ausubel et al. 1987).

In each of 4 rats, fresh cryostat sections of spinal cord 8 hr, 2 d and 7 d after nerve suture with electrical or sham stimulation were removed from the freezer (–80 C°). ISH of NFM, Tα1-tubulin and GAP-43 was carried out on 3 slides, the second, 8th and 14th, the 3rd, 9th and 15th slides, and the 4th, 10th and 16th of the total 8-20 for each probe, respectively. Each slide contained 6 sections at a distance of 450 µm along the longitudinal axis of the spinal cord. This provided sampling of motoneurons throughout the motoneuron pool along the 1500 µm longitudinal axis. ISH was carried out as described in detail by Verge et al. (1992). Briefly, spinal cord sections were hybridized to 10^6 c.p.m. of the probe in a 100 µl hybridization cocktail (deionised formamide, 20 x SSC, 50 x Denhardt's solution, 0.2 M sodium phosphate buffer, pH 7.0, dextran sulphate, 20 % sarcosyl). Control slides were hybridized as above in the presence of excess (400 x) unlabeled probe. After overnight hybridization at 42 C°, the slides were washed 4 times in 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, ph 7.0) for 15 min at 45 C°, rinsed briefly in a distilled water, and then dehydrated in ascending concentrations of ethanol to ensure that the sections adhere thereafter to the radiosensitive emulsion (Kodak NTP-2, diluted 1:1 in H_2O) during autoradiography. The sections were exposed to emulsion for adequate resolution of the silver grains activated by ^{35}S-ATP per probe in the dark for 1 week period for NFM, Tα1-tubulin and GAP-43 mRNA (Kobayashi et al., 1997; Fernandes et al., 1999). They were subsequently stained with neutral red, dehydrated, and embedded in Permount.

4.2.4 Quantification of ISH signals

Six to 7 motoneurons per section (~ 40 per slide; 30 motoneurons/rat) were randomly chosen for analysis in each of 4 rats. Neuronal cell bodies and nuclei were visualized and traced at 20-40x under UV fluorescence using barrier filters of 580 nM for FR and 430 nM for FG, and the area fraction occupied by silver grains (i.e., grain density) was then automatically measured using Image-Pro Plus (Media Cybernetics) from the corresponding dark-field image. Grain density, which represents mRNA per grain, was corrected by subtracting the background autoradiographic signal. For each motoneuron, the fraction of the area occupied by autoradiographic silver grains was multiplied by the total cell area and expressed autoradiographic signal. For each motoneuron, the fraction of the area occupied by autoradiographic silver grains was multiplied by the total cell area and expressed as a percentage of the signal. For each condition the mean percentage silver grain signal (±S.D.) was calculated. The investigator was unaware of the time point and which oligonucleotide probe had been applied to the slide.

4.2.5 Statistical analysis

A one-way ANOVA was used to compare the mean percentage silver grain signal (±S.D.) of the axotomized and the contralateral control motoneurons. A multi-factorial ANOVA was used to compare the mean percentage silver grain signal (±S.D.) among all time points. The same comparison was used for the stimulation. Statistical significance was set at the 0.05 level.

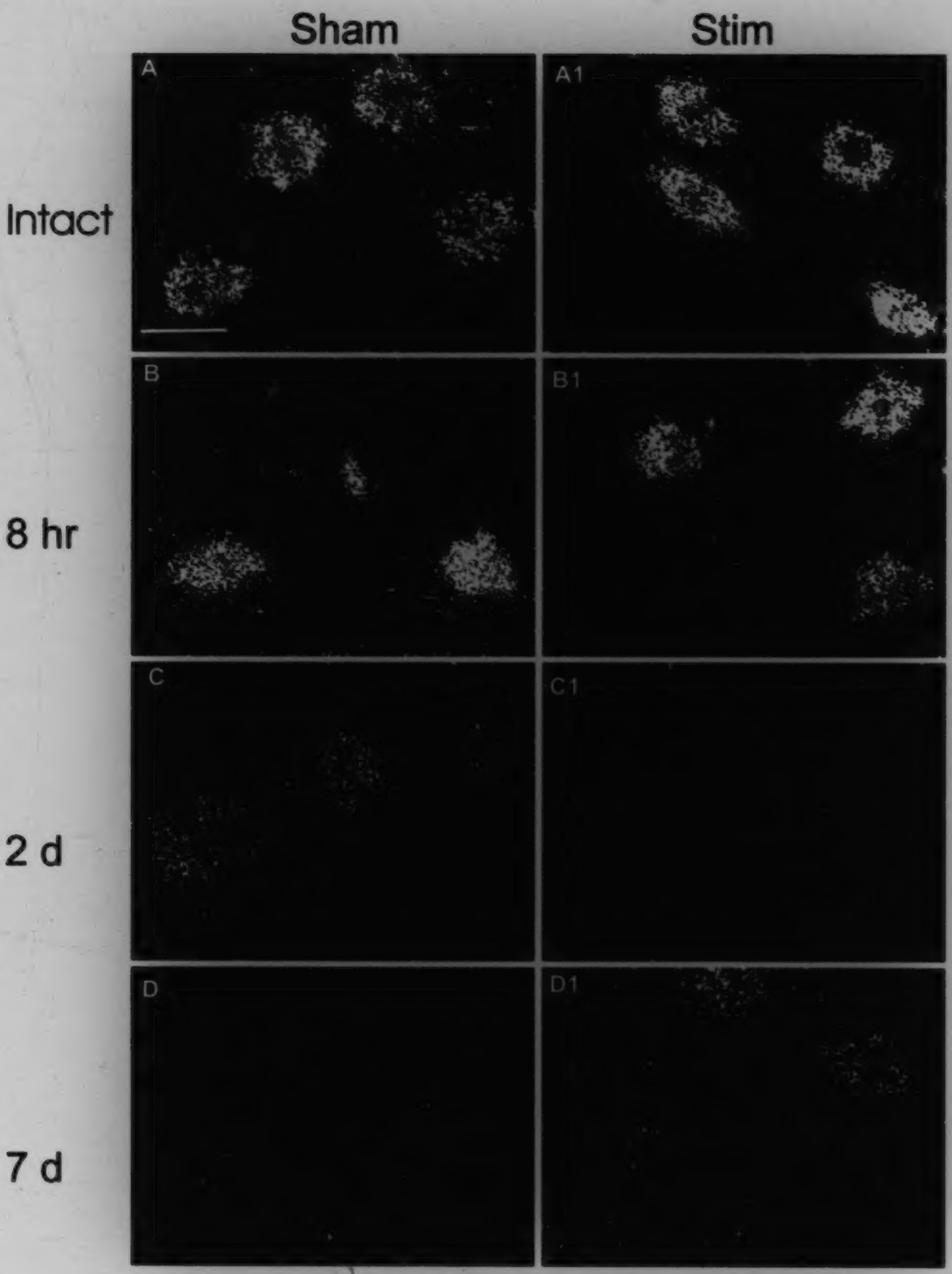
4.3 RESULTS

4.3.1 Expression of NFM mRNA in stimulated and sham-stimulated motoneurons after femoral nerve cut and suture

ISH with 1 week exposure of spinal cord cross-sections to emulsion for autoradiography (see methods), was used to semi-quantify NFM mRNA in regenerating motoneurons subjected to 1 hr 20 Hz or sham stimulation as well as in normal motoneurons on the contralateral side of the spinal cord. An example of ISH for NFM is displayed in Fig. 4.1. Control slides revealed no signal for NFM in motoneurons. High levels of NFM mRNA expression were seen in the intact femoral motoneurons (Fig. 4.1A,A1). Eight hours after femoral nerve transection and surgical suture with sham stimulation, the signals were similar to those in the contralateral intact side (Fig. 4.1A,B). Signals started to decrease at 2 d and the reduction was very obvious 7 d later (Fig. 4.1C,D)

When sutured nerves were subjected to 1 hr stimulation at 20Hz, silver grain densities decreased dramatically and earlier, 2 d after axotomy and nerve suture. In fact, 1 hr stimulation decreased silver grains densities at 2 d to a very low level just above background (Fig. 4.1C1). Moreover, the density of silver grains 2 d after nerve suture and stimulation was obviously lower than the sham stimulated motoneurons even at 7 d (cf. Fig. 4.1C1,D). At 7 d, the grain density in the stimulated regenerating motoneurons start to increase and reach the levels seen at 7 d in the sham-stimulated regenerating motoneurons (cf. Fig. 4.1D, D1).

FIGURE 4.1. Dark field micrographs of ISH with ^{35}S-labeled oligoneucleotide probe to detect NFM mRNA rat femoral motoneurons. Motoneurons were subjected either to *sham-stimulation* (**A**-**D**) or to *1 hr 20 Hz electrical stimulation* (**A1**-**D1**). **A**, **A1**. Contralateral (*intact*) femoral motoneurons. **B1**-**D1**. Femoral motoneurons, *8 hr* (**B, B1**), *2 d* (**C, C1**) and *7 d* (**D, D1**) after nerve cut and suture. Note the very high level expression of NFM mRNA in intact motoneurons (A, A1). Note also, the progressive decline of grain density in sham-stimulated femoral motoneurons (B-D) as compared to the earlier (2 d) and dramatic reduction seen in stimulated regenerating motoneurons (B1-D1). Scale bar *50 μm*


Sham
Stim
A
A1
Intact
B
B1
8 hr
C
C1
2 d
D
D1
7 d

Quantification of the ISH signal per cell profile revealed a relatively very high NFM ISH signal (NFM mRNA levels) in intact motoneurons sampled from unoperated side of all rats (Fig. 4.2A). The NFM ISH signal/cell profile showed the same normal distribution 8 hr after femoral nerve suture and sham stimulation and was not statistically different from the contralateral intact side ($P > 0.05$, Fig. 4.1B). Mean (± SE) values for the cellular area occupied by silver grain were 59 ± 0.8 and 58.1 ± 0.9 for intact controls for sham and stimulated groups respectively and 56.3 ± 0.8 for 8 hr after nerve suture and sham stimulation. As expected, ISH signal for NFM mRNA decreased in regenerating motoneurons in the first week of regeneration. The NFM ISH signals decreased to significantly lower levels by 2 d (39 ± 0.7, $P < 0.05$) and dropped further by 7 d (15.1 ± 0.4, $P < 0.01$) (Fig. 4.3C,D).

When regenerating motoneurons were subjected to 1 hr electrical stimulation after nerve section and suture, there was a dramatic decrease in the mean values of the NFM ISH signal by 2d (5.5 ± 0.2 , $P < 0.01$, Fig. 4.2C). This value was ~ 12 and ~8 times lower than ISH signals for NFM found in intact control motoneurons, and in regenerating motoneurons 2 after nerve cut, suture and sham stimulation, respectively. As shown in Fig. 4.2, the histograms for the NFM ISH signals in the stimulated motoneurons were shifted far to the left of the signals in the intact control motoneurons and sham-stimulated motoneurons 2 d after nerve suture and stimulation. At 7 d, when motoneurons were still regenerating their axons, NFM ISH mRNA levels began to increase slowly to reach levels significantly higher than levels seen at 2 d. Relative NFM ISH mRNA levels in sham-stimulated axotomized motoneurons were not significantly different than the levels in the stimulated motoneurons (Fig. 4.2D). The consistency in the data among animals is seen in Fig. 4.3 where the mean (± SD) for at least 30 motoneurons is compared for 4 animals at each time point in the stimulated and sham-stimulated groups. The NFM ISH signal decreased ~12-fold by 2 d after electrical stimulation, in contrast to slightly less than a 2-fold decrease by 2 d in the sham-stimulated group.

FIGURE 4.2. Frequency histograms of NFM ISH signal/cell profile (mRNA expression per motoneuron) in regenerating femoral motoneurons subjected to *sham-stimulation* (*open histograms*) or to *1 hr continuous 20 Hz frequency electrical stimulation* (*stim, filled histograms*). **A**, Contralateral (*intact*) femoral motoneurons and regenerating motoneurons *8 hr* (**B**), *2 d* (**C**) and *7 d* (**D**) after femoral nerve cut and surgical suture. Note that intact motoneurons express very high levels of NFM mRNA, which remain at the same level, 8 hr after axotomy, and nerve suture, until the levels are progressively reduced at 2 and 7 days in sham-stimulated regenerating motoneurons (*open bars*). This contrasts with a rapid and great reduction of NFM mRNA expression 2 d after electrical stimulation (*filled bars*). Electrical stimulation elevates the mean mRNA expression in regenerating motoneurons as well as decrease the range of ISH signal /cell values.

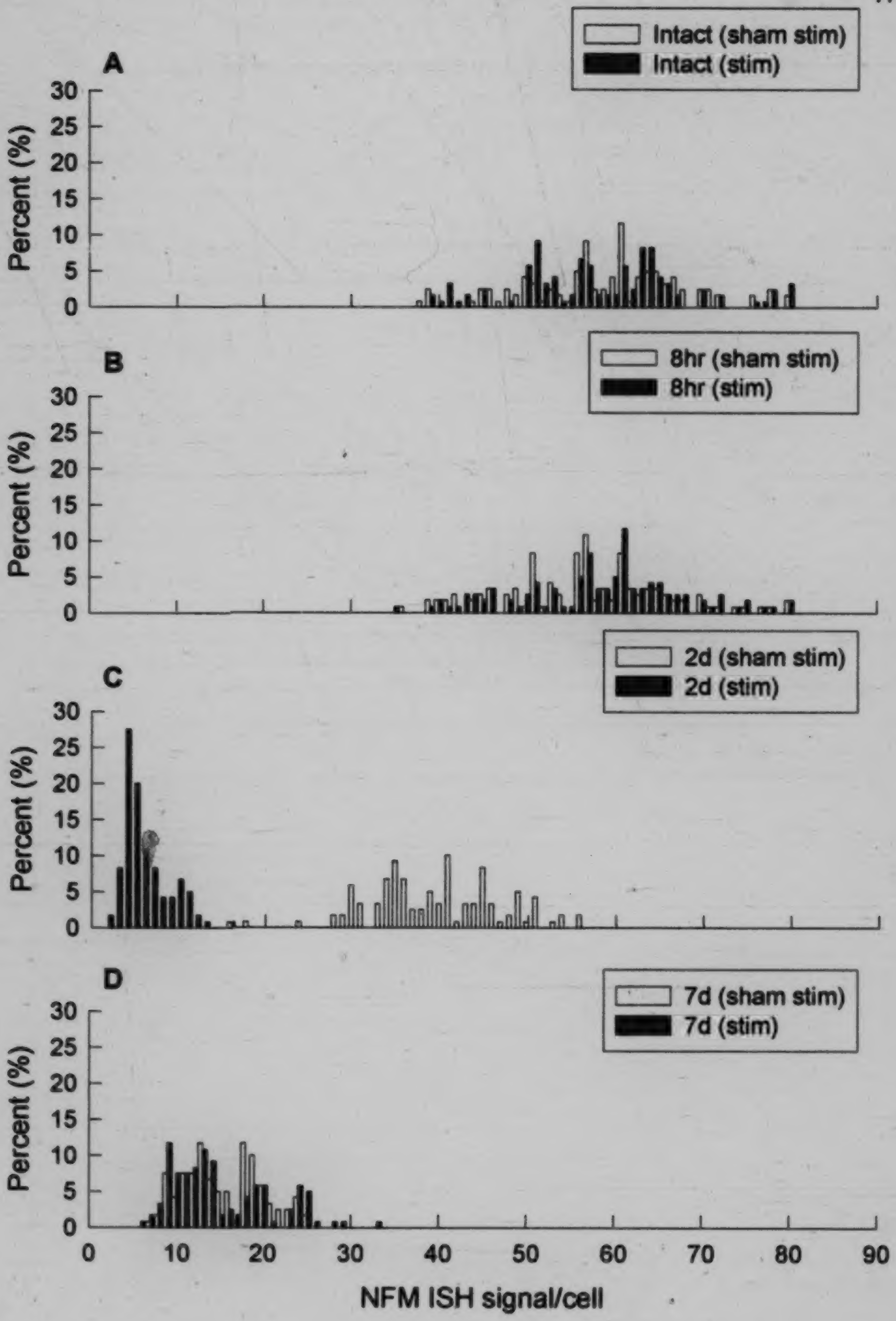
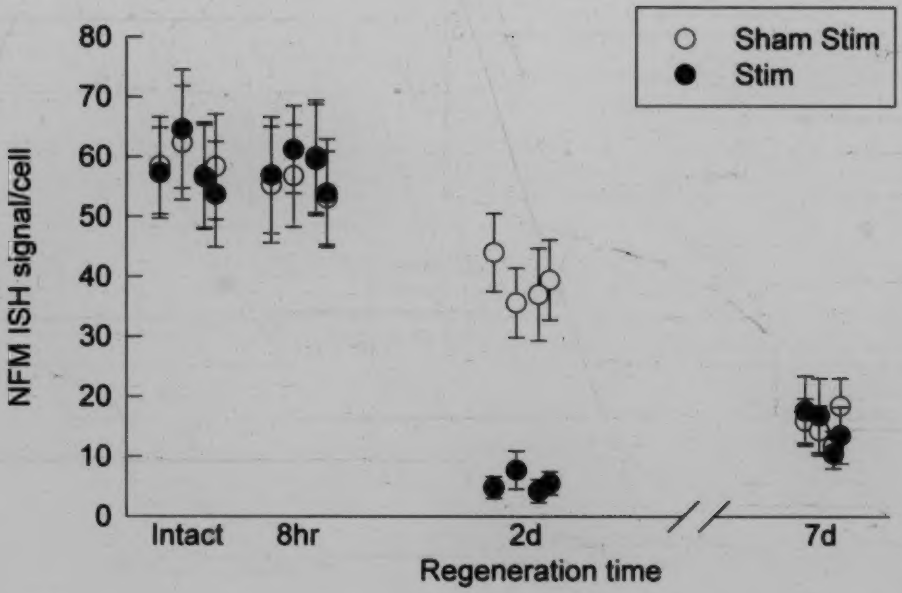

A
Intact (sham stim)
Intact (stim)
B
8hr (sham stim)
8hr (stim)
C
2d (sham stim)
2d (stim)
D
7d (sham stim)
7d (stim)
Percent (%)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
90
NFM ISH signal/cell

FIGURE 4.3. The dramatic and rapid reduction of motoneuron expression of NFM mRNA in response to 1 hr 20 Hz electrical stimulation of axotomized and surgically sutured femoral motoneurons. The mean ± SD of the NFM ISH signal/cell is plotted for motoneurons subjected to *sham-stimulation* (*open symbols*) and *electrical stimulation* (*filled symbols*) vs. survival time (*8 hr, 2 d and 7 d*) after femoral nerve and suture. Note that the mean ± SD of the values obtained for 4 different rats per time point are displayed along the time-axis for clarity. The mean values of ISH signal/cell profile in individual rats for each time point were relatively similar in both sham-stimulated and stimulated motoneurons. Backfilling of motoneurons alternatively with either FG or FR in the group of 4 rats per time point, demonstrates that the retrograde labeling with either dye did not affect mRNA expression. Note also the decrease in variability around the mean values for the stimulated motoneurons as shown by the smaller SDs.



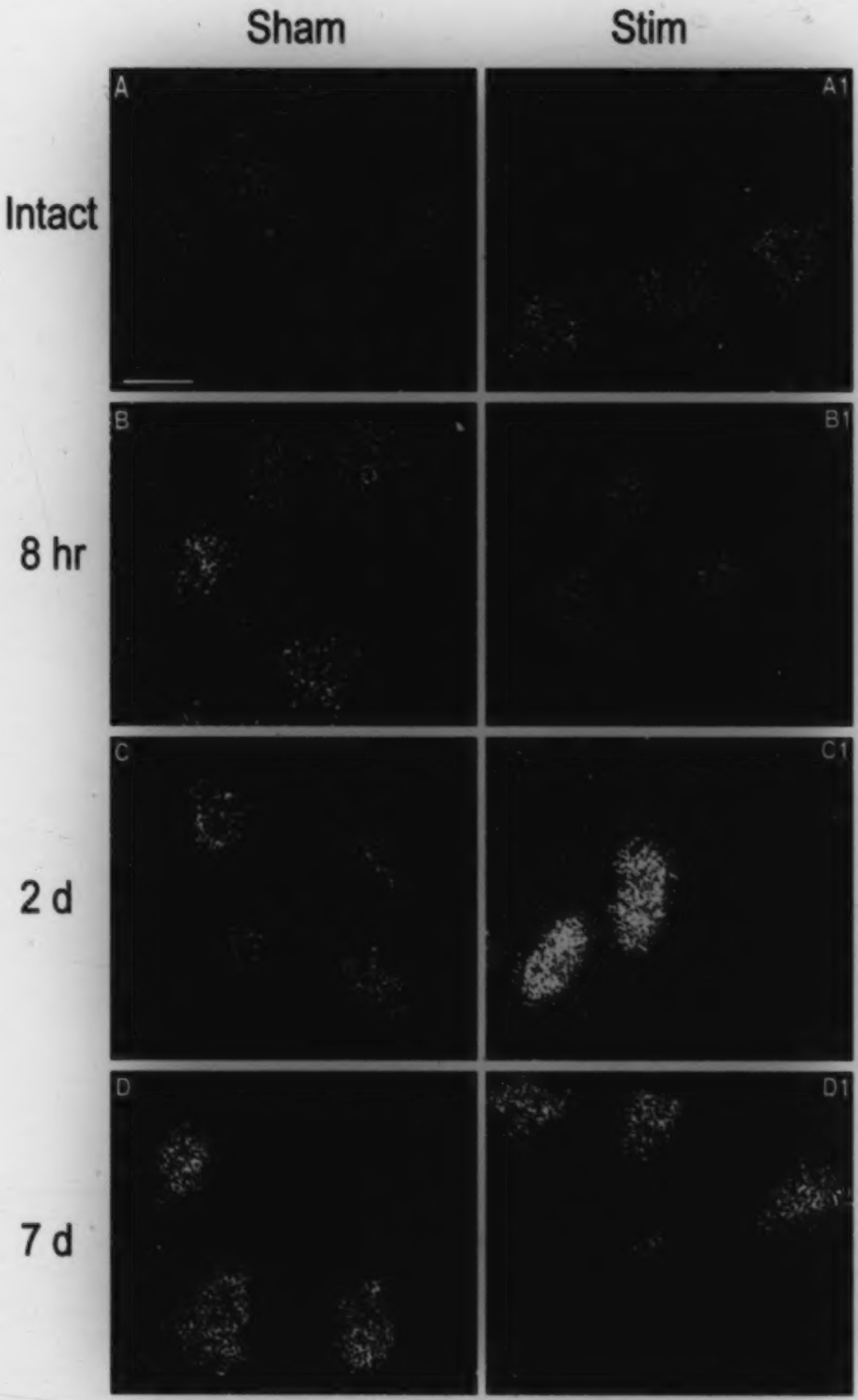
NFM ISH signal/cell
0
10
20
30
40
50
60
70
80
Intact
8hr
2d
7d
Regeneration time
○ Sham Stim
● Stim

4.3.2 Expression of Tα1-tubulin mRNA in stimulated and sham-stimulated motoneurons after femoral nerve cut and suture

The hypothesis that stimulation induced acceleration of motor axonal regeneration is due to rapid downregulation of NFM concurrent with upregulation of Tα1-tubulin mRNA and, in turn, a decrease in neurofilament/tubulin mRNA ratio, predicts that stimulation will accelerate and upregulate the expression of Tα1-tubulin in the same motoneurons in which NFM was downregulated. We used an oligonucleotide probe which specifically recognises Tα1-tubulin (Kobayashi et al., 1997; Fernandes et al., 1999) to investigate the relative change in expression of Tα1-tubulin in sham-stimulated and 20 Hz stimulated motoneurons, 8 hr, 2 d and 7 d after femoral nerve cut and suture. ISH with 1 week exposure of spinal cord cross-sections to emulsion for autoradiography, was used to semi-quantify Tα1-tubulin mRNA expression. A direct comparison of the Tα1-tubulin signal/cell in axotomized and sutured femoral motoneurons relative to motoneurons in the contralateral side of the same animal is shown in Fig. 4.4. Control slides revealed no signal for Tα1-tubulin in motoneurons. Contralateral uninjured motoneurons showed distinct signals for Tα1-tubulin mRNA (Fig. 4.4A,A1). The expression of the Tα1-tubulin was not elevated 8 hr and 2 d after femoral nerve cut and suture (Fig. 4.4B,C), but the Tα1-tubulin ISH signal/cell in axotomized and sutured motoneurons was increased by 7 d after nerve cut and suture (Fig. 4.4D).

The effect of 1 hr electrical stimulation at 20 Hz on Tα1-tubulin mRNA level in regenerating motoneurons was dramatic demonstrating the same time dependent increase in Tα1-tubulin as shown in Fig.4.1 for the decline in NFM. Silver grain densities increased dramatically and earlier, 2 d after axotomy and nerve suture (Fig. 4.4C1). In fact, the very high density of silver grains, at 2 d, clearly delineates the stimulated motoneurons from background staining, even without visualization of the retrograde FG or FR labels. Moreover, the density of silver grains at 2 d after nerve suture and stimulation was obviously higher than the unstimulated motoneurons even at 7 d (cf. Fig. 4.4C1,D). By 7 d, the grain density in the stimulated regenerating motoneurons declined to levels seen at 7 d in the sham-stimulated regenerating motoneurons (cf. Fig. 4.4D, D1).

FIGURE 4.4. Dark field micrographs of ISH with ^{35}S-labeled oligoneucleotide probe to detect Tα1-tubulin mRNA in rat femoral motoneurons. Motoneurons were subjected either to *sham-stimulation* (**A-D**) or to *1 hr 20 Hz electrical stimulation* (**A1-D1**). **A**, **A1**. Contralateral (*intact*) femoral motoneurons. **B1-D1**. Femoral motoneurons, *8 hr* (**B, B1**), *2 d* (**C, C1**) and *7 d* (**D, D1**) after nerve cut and suture. Note the delayed (7 d) increase in grain density of Tα1-tubulin mRNA in sham-stimulated regenerating motoneurons (A, A1, B-D) as compared to the earlier (2 d) increases seen in stimulated regenerating motoneurons (C1). Scale bar *50 μm*


Sham
Stim
Intact
8 hr
2 d
7 d
A
A1
B
B1
C
C1
D
D1

The frequency distributions of Tα1-tubulin ISH signals/cell profile in the axotomized and surgically sutured motoneurons are compared for the sham-stimulated and 1 hr stimulated regenerating motoneurons in Fig. 4.5. Quantification of the autoradiographic silver grains (signal/cell) revealed limited variability in the Tα1-tubulin ISH signal in intact motoneurons sampled from unoperated side of all rats (Fig. 4.5A). The Tα1-tubulin ISH signal/cell profile showed the same normal distribution 8 hr and 2 d after femoral nerve suture and sham stimulation which was not statistically different from the contralateral intact side ($P > 0.05$, Fig. 4.5B,C). Mean (± SE) values for the cellular area occupied by silver grain were 20.3 ± 0.3 and 21.1 ± 0.4 for intact controls for sham and stimulated groups respectively and 22 ± 0.4 and 24 ± 0.4 for 8 hr and 2 d after nerve suture and sham stimulation, respectively. The Tα1-tubulin ISH signal increased to significantly higher levels by 7 d (39.3 ± 0.7, $P < 0.01$).

In contrast to unchanged levels of mRNA at 2 d with sham-stimulation relative to contralateral intact motoneurons, there was a dramatic increase in the mean values of the Tα1-tubulin ISH signal in the femoral motoneurons 2 d after nerve section and suture and 1 hr electrical stimulation (62.1 ± 0.9, $P < 0.01$). As shown in Fig. 4.5, the histograms for the Tα1-tubulin ISH signal in the stimulated motoneurons were shifted to higher values far to the right of the signals in the sham-stimulated motoneurons 2 d after nerve suture. At 7 d, Tα1-tubulin mRNA levels were relatively the same as the sham-stimulated axotomized and surgically sutured motoneurons (Fig. 4.5D). Similar to NFM data there was a consistency in the data among animals (Fig. 4.6). The Tα1-tubulin ISH signal increased 3.1-fold by 2 d after electrical stimulation, in contrast to slightly less than a 2-fold increase by 7 d in the sham-stimulated group. These data, show a parallel acceleration and elevation in expression of Tα1-tubulin and rapid and dramatic down regulation of NFM in electrically stimulated axotomized motoneurons which regenerate their axons. NFM/ Tα1-tubulin expression ratio was 1.6 to 1 in sham stimulated motoneurons 2 d after nerve repair. This ratio was dramatically reduced by ~ 18-fold to reach 1 to 11.3 when motoneurons were subjected to 20 Hz continuous electrical stimulation for 1 hr. These results support the hypothesis that concurrent upregulation of Tα1-tubulin with rapid downregulation on neurofilament plays a central role in the acceleration of motor axonal regeneration by short-term electrical stimulation.

FIGURE 4.5. Frequency histograms of Tα1-tubulin ISH signal/cell profile (mRNA expression per motoneuron) in regenerating femoral motoneurons subjected to *sham-stimulation* (*open histograms*) or to *1 hr continuous 20 Hz frequency electrical stimulation* (*stim, filled histograms*). **A**, Contralateral (*intact*) femoral motoneurons and regenerating motoneurons *8 hr* (**B**), *2 d* (**C**) and *7 d* (**D**) after femoral nerve cut and surgical suture. Note that intact motoneurons express detectible levels of Tα1-tubulin mRNA which remain at the same level, 8 hr and 2 d after axotomy and nerve suture, until the levels are ~ doubled at 7 days in sham stimulated regenerating motoneurons (*open bars*). This contrasts with an accelerated and greatly enhanced Tα1-tubulin mRNA expression 2 d after electrical stimulation (*filled bars*). Electrical stimulation elevates the mean mRNA expression in regenerating motoneurons as well as increases the range of ISH signal /cell profile values.

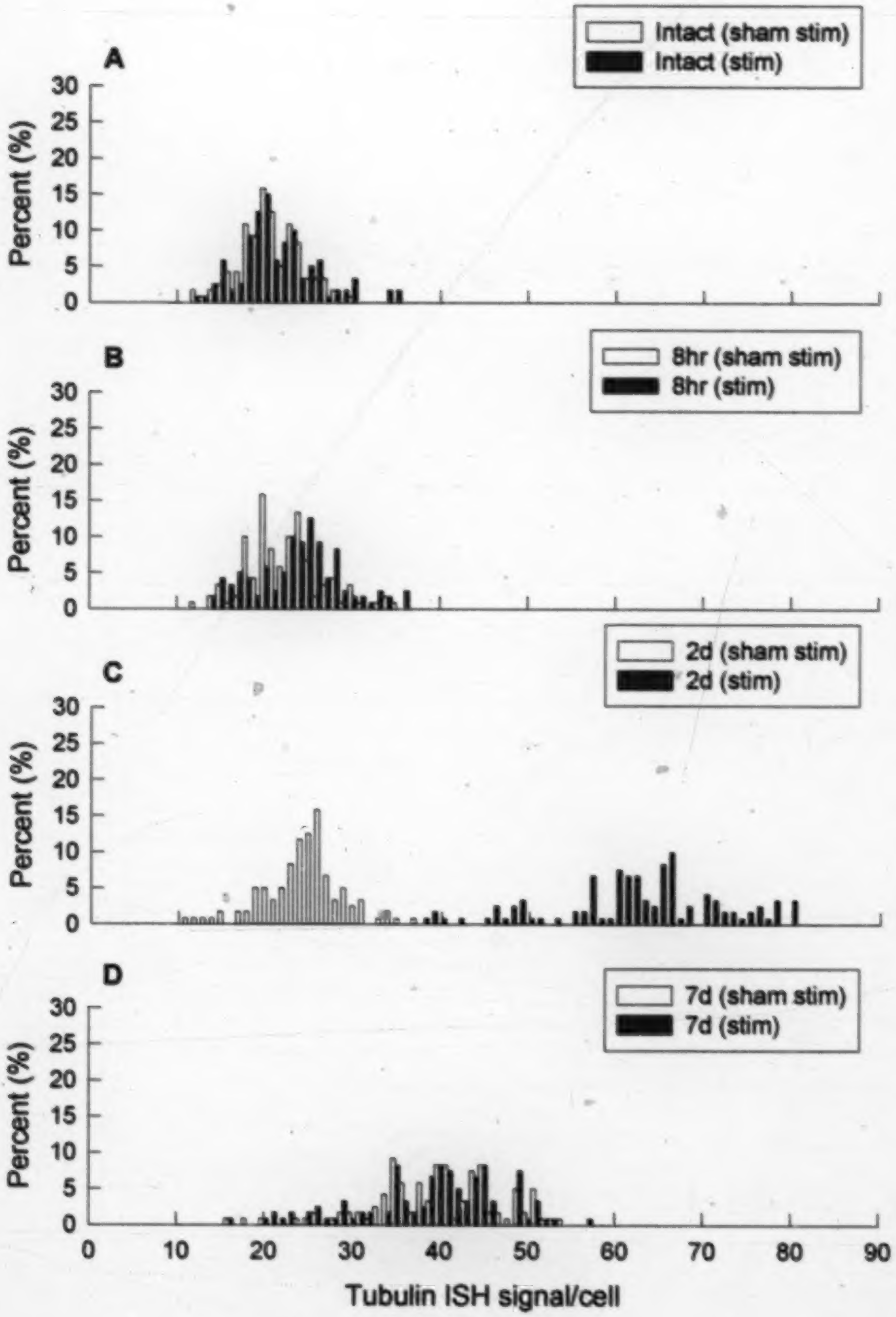
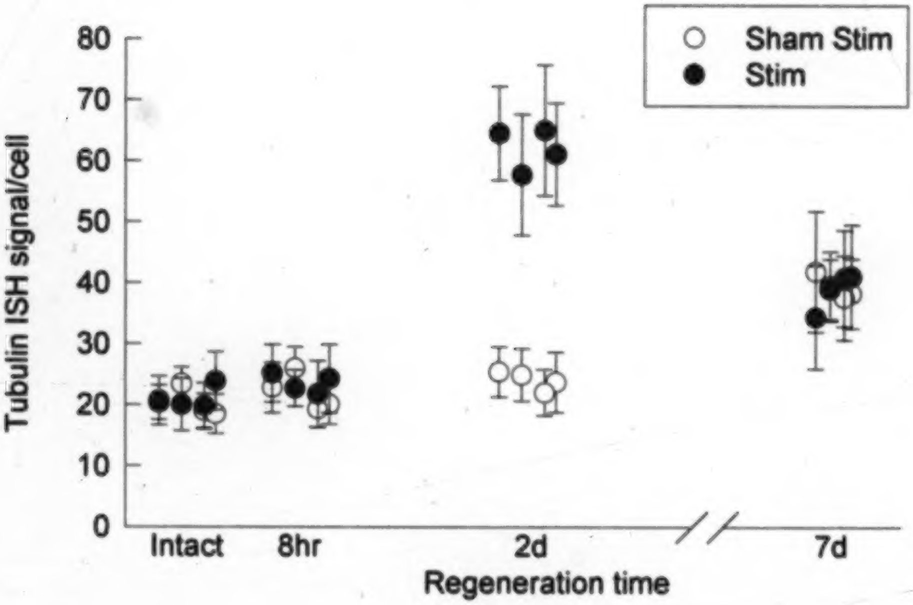

A
Intact (sham stim)
Intact (stim)
B
8hr (sham stim)
8hr (stim)
C
2d (sham stim)
2d (stim)
D
7d (sham stim)
7d (stim)
Percent (%)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
90
Tubulin ISH signal/cell

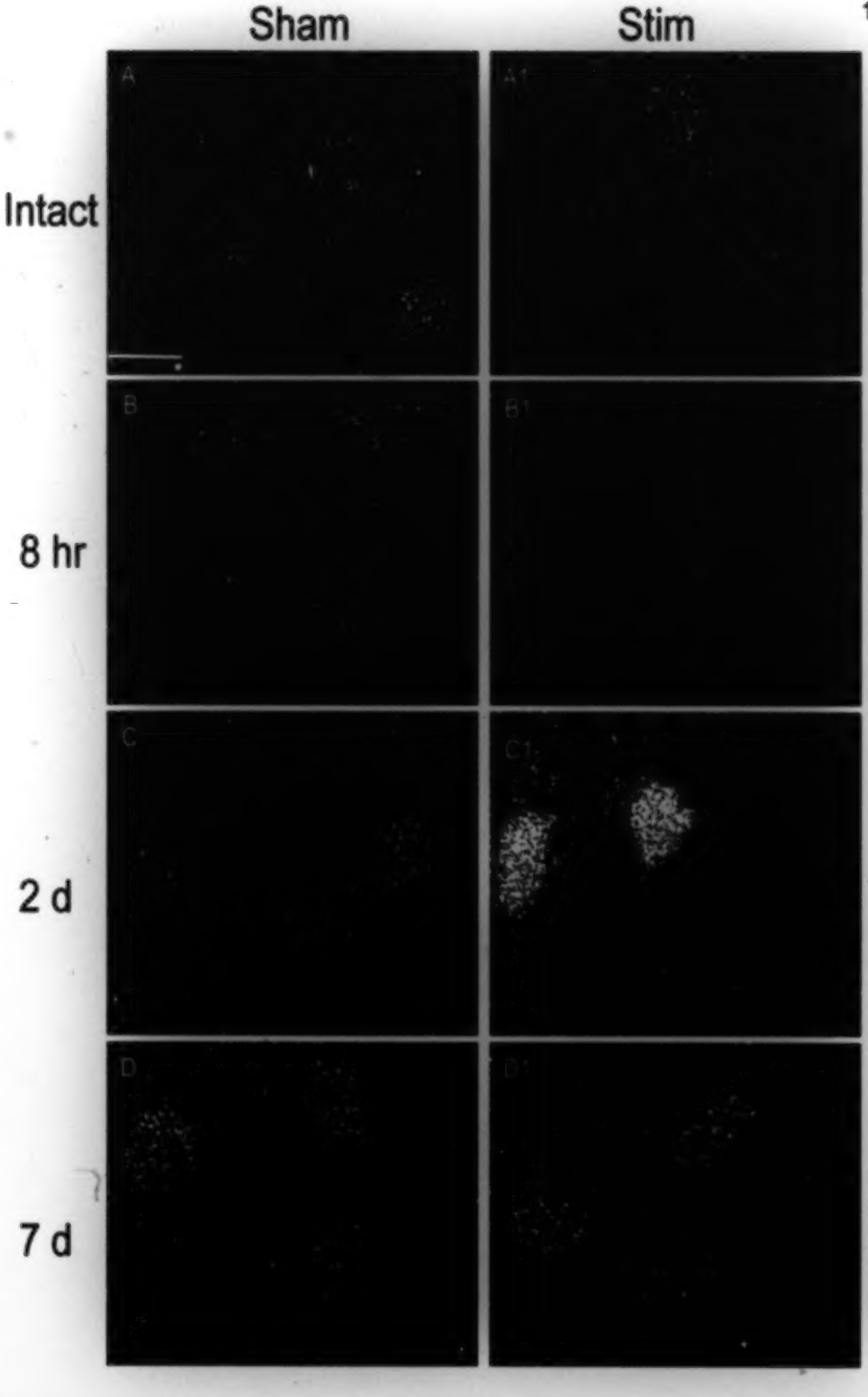
FIGURE 4.6. The acceleration and elevation of motoneuron expression of Tα1-tubulin mRNA in response to 1 hr 20 Hz electrical stimulation of axotomized and surgically sutured femoral motoneurons. The mean ± SD of the Tα1-tubulin ISH signal/cell profile is plotted for motoneurons subjected to *sham-stimulation* (*open symbols*) and *electrical stimulation* (*filled symbols*) vs. survival time (*8 hr, 2 d and 7 d*) after femoral nerve and suture. The mean values of ISH signal/cell profile in individual rats for each time point were relatively similar in both sham-stimulated and stimulated motoneurons. Note also the increase in variability around the mean values for the stimulated motoneurons as shown by the larger SDs.


Sham Stim
Stim
Tubulin ISH signal/cell
0
10
20
30
40
50
60
70
80
Intact
8hr
2d
7d
Regeneration time

4.3.3 Expression of GAP-43 mRNA in stimulated and sham-stimulated motoneurons after femoral nerve cut and suture

For GAP-43 ISH, a probe was used which specifically recognized GAP-43 mRNA (Basi et al., 1987; Kobayashi et al. 1997; Fernandes et al. 1999). A direct comparison of the quantification of the GAP-43 ISH signal/cell profile in axotomized and sutured femoral motoneurons relative to motoneurons in the contralateral side of the same animal is shown in Fig. 4.7. Control slides revealed no signal forGAP-43 in motoneurons Contralateral uninjured intact femoral motoneurons showed low but detectable levels of GAP-43 mRNA after 1 week of autoradiographic exposure (Fig. 4.7A,A1). As demonstrated for Tα1-tubulin mRNA, there was a delayed upregulation of GAP-43 mRNA in sham-stimulated motoneurons in contrast to a rapid upregulation by 2 d after 1 hr stimulation (Fig. 4.5B-C, B1-C1). By 7 d after nerve suture, the expression of GAP-43 was similar in sham-stimulated and 20 Hz stimulated motoneurons (Fig. 4.7D,D1).

FIGURE 4.7. Dark field micrographs of ISH with ^{35}S labeled oligoneucleotide probe to detect GAP-43 mRNA in rat intact and regenerating femoral motoneurons which are subjected to either *sham-stimulation* (**A**-**D**) or to *1 hr 20 Hz electrical stimulation* (**A1**-**D1**). **A**, **A1**, Contralateral (*intact*) femoral motoneurons. **B1**-**D1**, Femoral motoneurons *8 hr* (**B, B1**), *2 d* (**C, C1**) and *7 d* (**D, D1**) after nerve cut and suture. Note the low level expression of GAP-43 mRNA in intact motoneurons and the delay in upregulation of GAP-43 in the sham-stimulated regenerating motoneurons until 7 d after surgical suture similar to that found for Tα1-tubulin as shown in Figure 4. Note also the earlier and obviously greater elevation of GAP-43 signals after electrical stimulation of the regenerating motoneurons, as found for Tα1-tubulin in the same motoneurons. However, the magnitude of change in gene expression (calculated by comparing with intact motoneurons) of GAP-43 and Tα1-tubulin appeared to be more for GAP-43 than Tα1-tubulin at 2 d (cf A1, C1 in Fig. 4.4). Scale bar *50 μm*


Sham
Stim
Intact
8 hr
2 d
7 d
A
A1
B
B1
C
C1
D
D1

The histograms of GAP-43 ISH quantification obtained from representative motoneurons are shown in Fig. 8. The effect of 1 hr 20 Hz electrical stimulation in accelerating the upregulation GAP-43 mRNA was similar to the effect of stimulation on Tα1-tubulin mRNA. However, electrical stimulation amplified GAP-43 expression to a greater extent than Tα1-tubulin mRNA expression. This is seen as a relatively greater rightward shift of the histograms along the GAP-43 ISH signal axis at 2 d and a 7.4-fold increase in the mean values by 2 d in contrast to the 3.1-fold increase in Tα1-tubulin expression at 2 d (cf. Figs. 4.9 and 4.6). However, the 2-fold elevation of GAP-43 mRNA signals at 7 d for the stimulated and sham-stimulated motoneurons (Fig. 4.9) was the same as the 2-fold increase seen for Tα1-tubulin mRNA at the same time after femoral nerve section and nerve suture (Fig. 4.6). These results indicated that 1 hr electrical stimulation significantly increased RAGs (Tα1-tubulin and GAP-43 mRNA) during the first week of regeneration.

FIGURE 4.8. Frequency histograms of GAP-43 ISH signal/cell profile of intact femoral motoneurons and axotomized and surgically sutured femoral nerves subjected to *sham-stimulation* (*open histograms*) and to *1 hr 20 Hz electrical stimulation* immediately after surgical suture (*filled histograms*). **A**, Contralateral (*intact*) femoral motoneurons, and regenerating motoneurons *8 hr* (**B**), *2 d* (**C**) and *7 d* (**D**) after nerve cut and surgical suture. Note that intact motoneurons express low levels of GAP-43 mRNA which remain at the same level, 8 hr and 2 d after axotomy and nerve suture, until the levels are doubled at 7 days in sham stimulated regenerating motoneurons (*open bars*). Note that electrical stimulation of axotomized and surgically sutured femoral motoneurons accelerates and enhances mRNA expression of the GAP-43 as for the cytoskeletal protein Tα1-tubulin shown in Fig. 4.4. However, the relative elevation in GAP-43 mRNA is more than for Tα1-tubulin (cf. 4.4A,C and 4.8A,C). Note the same scales on the X-axis in both figures.

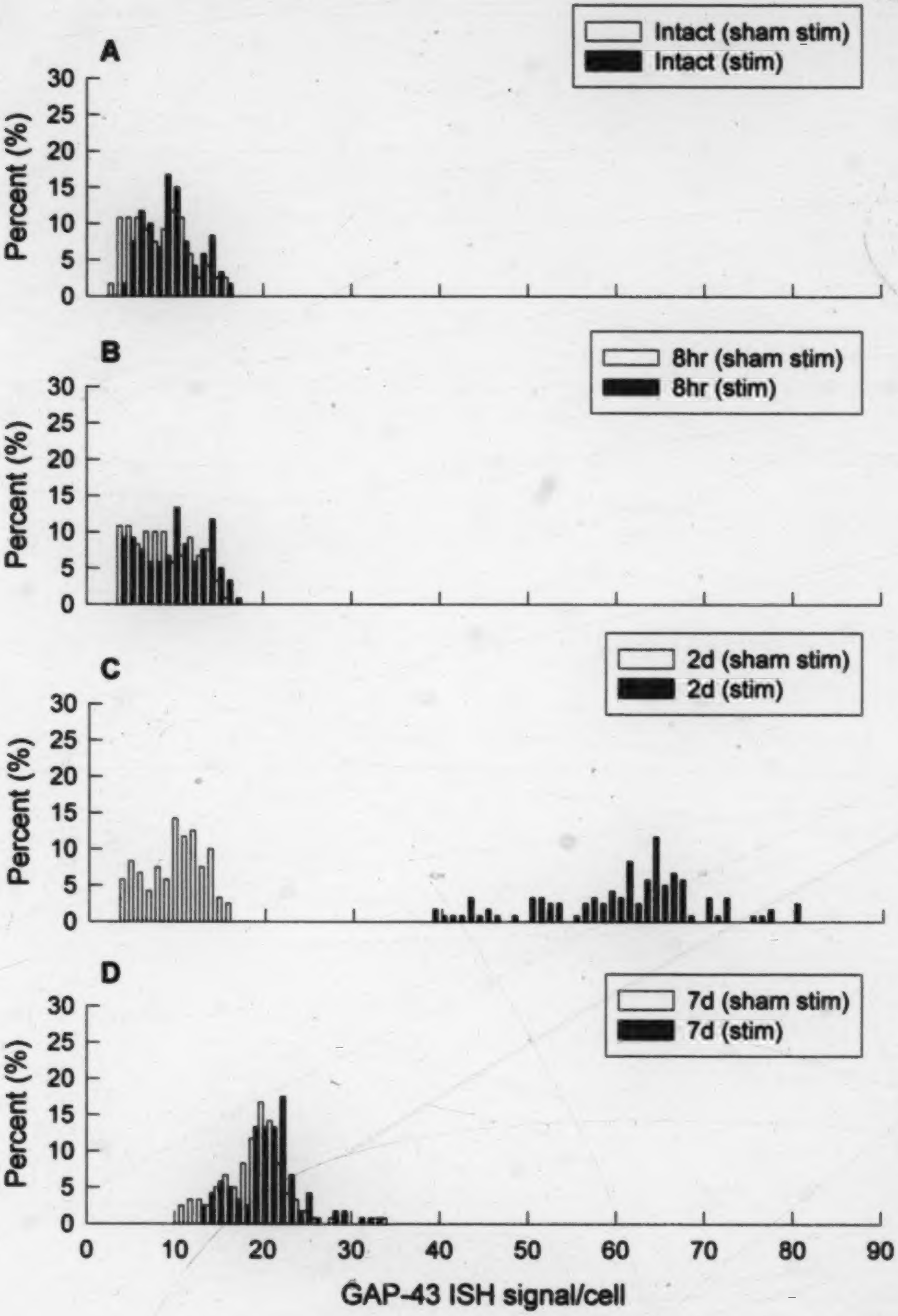
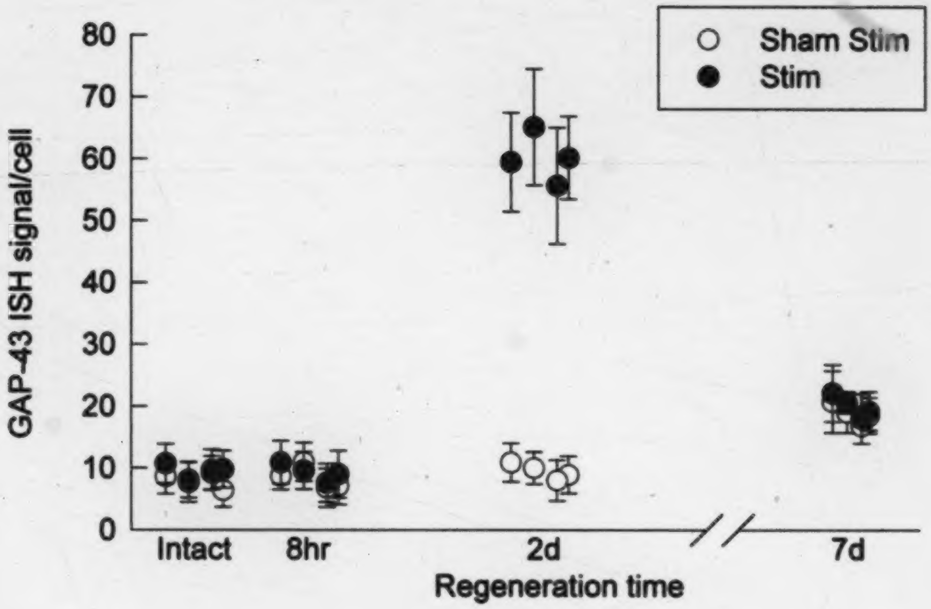

A
Intact (sham stim)
Intact (stim)
B
8hr (sham stim)
8hr (stim)
C
2d (sham stim)
2d (stim)
D
7d (sham stim)
7d (stim)
Percent (%)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
90
GAP-43 ISH signal/cell

FIGURE 4.9. The mean ± SD of the ISH signal/cell (mRNA expression) detecting GAP-43 mRNA in *intact* and regenerating femoral motoneurons, *8 hr*, *2 d* and *7 d* after *1 hr sham-stimulation* (*open symbols*) and *20 Hz stimulation* (*filled symbols*). The mean ± SD values are separated at each time point on the X-axis for comparison between data obtained from individual rats. Electrical stimulation of axotomized motoneurons upregulated mRNA expression of GAP-43 earlier and more dramatically than sham-stimulation. Note the larger relative increase of GAP-43 expression as compared to Tα1-tubulin expression in Fig. 4.5.


GAP-43 ISH signal/cell
0
10
20
30
40
50
60
70
80
Intact
8hr
2d
7d
Regeneration time
○ Sham Stim
● Stim

4.5 DISCUSSION

In this study, we used semi-quantitative ISH to demonstrate that short-term (1hr) continuous 20 Hz stimulation dramatically decreased mRNA levels for NFM concurrent with a large acceleration in the expression of genes for Tα1-tubulin and GAP-43 in regenerating motoneurons. These findings support the hypothesis that rapid reduction in NF/tubulin expression ratio by electrical stimulation in regenerating motoneurons plays a key role in the acceleration of axonal outgrowth by electrical stimulation (Al-Majed et al., 2000a) (Chapter 2).

4.5.1 Changes in NFM and Tα1-tubulin mRNA and axonal regeneration

In this study we have demonstrated that axotomized femoral motoneurons exhibit reduced ISH signals with NFM probes and increase their ISH signals when probed with oligonucleotides specific for Tα1-tubulin and GAP-43 during the first week after axotomy and surgical suture. Our findings are consistent with previous findings in axotomized but not repaired rat facial motoneurons, rubrospinal neurons (Tetzlaff et al., 1991) and sciatic motoneurons (Petrov et al., 1996). Tetzlaff et. al. (1991), using ISH, reported that axotomy of adult facial motoneurons stimulates upregulation of the RAGs, Tα1-tubulin and GAP-43 mRNA and downregulation of NFM; reaching peak and minimal levels, respectively, at 7 days. Tetzlaff et al (1991), also demonstrated that quantitative ISH data are in good agreement with quantitative northern blot analysis and with previously published ^{35}S-methionine incorporation measurements of protein levels (Tetzlaff et al., 1988). These findings emphasize that our technique of ISH consistently detects altered mRNA expression and is a suitable tool to analyze changes in gene expression in motoneurons within the complexity of neuronal circuitry of the CNS (spinal cord). Our findings here demonstrate for the first time that NFM, Tα1-tubulin and GAP-43mRNA expression are downregulated and upregulated respectively by axotomy under conditions when axonal regeneration is encouraged by surgical suture rather than discouraged by nerve transection (Tetzlaff et al., 1991).

In this study, where the femoral nerve transection and surgical suture model is used, ISH signals demonstrate that intact femoral motoneurons express mRNA encoding for NFM and Tα1-tubulin (Fig. 4.1A and 4.4A) as do other motoneurons (Tetzlaff et al., 1991; Pertov et al, 1996). The NFM/Tα1-tubulin expression ratio in intact femoral motoneurons is ~ 2.9 to 1 (Fig. 4.3A and 4.6) similar to what Tetzlaff et al (1991) had reported. This ratio decreased and almost reversed (~1 to 2.6) 7 d after nerve cut and suture, since regenerating motoneurons downregulate NFM and upregulate Tα1-tubulin (Fig. 1D, 4.3,4.4D, and 4.6). Note that the decrease in NFM occurs earlier than the increase in Tα1-tubulin (2d after nerve repair and suture, see Fig. 4.1B,4.2c and 4.3), which is also show as an initial shift in the NFM/ Tα1-tubulin ratio. This may reflect earlier requirements for decreased NFM synthesis during regeneration, perhaps related to the facilitation of axonal transport of increased levels of Tα1-tubulin in the SCb component of slow axonal transport. The SCb axonal transport rate of tubulin closely correlates with rate of axonal elongation (1-3 mm/d) in regenerating rat nerves suggesting that the slow transport of tubulin determines rate of elongation of regenerating axons (McQuarrie, 1983; Wujek and Lasek, 1983). Lasek and Katz (1987) proposed that axonal elongation is achieved by an elongation of the axonal cytoskeleton which is synthesized and assembled at the cell body and which then moves down the axon either as a continuous network or as individual polymers to supply cytoskeleton to extend the regenerating axons (Lasek, 1986). The differential changes in the synthesis of cytoskeletal proteins, neurofilament and tubulin in motoneurons may result in significant changes in their normal interaction in axons.

In intact motoneurons, tubulin interacts with neurofilaments whose number correlates with axonal size (Ellisman and porter, 1980; Shelanski et al., 1981; Tashiro et al., 1984). The majority of the tubulin migrates at the same velocity of axonal transport as the neurofilament proteins (Hoffman and Lasek, 1975; Oblinger et al., 1987). In regenerating motoneurons, the reduction in neurofilaments correlates with reduced axonal diameter and may result in fewer neurofilament-tubulin interactions. As a result a greater proportion of tubulin might travel at a higher velocity. This is indeed the case since during regeneration a greater portion of the tubulin is transported at a higher velocity (SCb axonal transport) (Hoffman and Lasek, 1980; Hoffman et al., 1985). This high proportion of tubulin travelling with SCb may be a determinant of axonal elongation (McQuarrie, 1983), and a correlation between SCb velocity and axonal regeneration rate has been reported (Wujek and Lasek, 1983). Motoneurons regenerate over a protracted period of up to 10 weeks as they progressively reinnervate appropriate muscle pathways (Al-

Majed et al., 2000a) (Chapter 2). The relative delay in the upregulation of Tα1-tubulin in the femoral nerve model, in addition to the fact that the reduction in NFM synthesis at the cell body would take many days to affect the NFM content of distal axons (Hoffman et., 1987), could thus contribute to the prolonged time required for all motoneurons to regenerate their axons over a 25 mm distance (Al-Majed et al., 2000a) (Chapter 2).

4.5.2 Changes in GAP-43 mRNA and axonal regeneration

Upregulation of GAP-43 after femoral nerve cut and suture is consistent with the "GAP hypothesis" (for review, see Skene, 1989), which predicts that the expression of growth-associated proteins, such as GAP-43, is a prerequisite to successful axonal regeneration and that failure to express these genes by CNS neurons after injury relates to their failure to regenerate. For GAP-43, the correlation between its expression and capacity to regenerate has been reported in a variety of examples in the PNS (for review, see Skene, 1989, 1992; Caroni, 1997). For example, it has been recently demonstrated that DRG axons growing within the severed dorsal root consist of a rapidly regenerating population which demonstrate a high baseline GAP-43 expression and a slowly growing population which have correspondingly lower levels of GAP-43 (Andersen and Schreyer, 1999). GAP-43 has been characterized as a brain specific substrate for protein kinase C (Aloyo et al., 1983; Karns et al., 1987). It appears to play a critical role in axonal growth cone function (for review, see Skene, 1989, 1992; Aigner and Caroni, 1993;

Dent and Meiri, 1998; Meiri et al., 1998). It has been proposed that GAP-43 is involved in the second messenger systems in the growth cone that are activated by various exogenous stimuli and that its function in membrane signal transduction is important for initiation of sprouting and membrane plasticity (Lookeren et al., 1992). Gene deletion of GAP-43 in knockout mice perturbs axonal pathfinding, thus confirming an important function for it within the growth cone (Strittmatter et al., 1995; Kruger et al., 1998; Sretavan and Kruger, 1998).

4.5.3 Effects of 1 hr electrical stimulation on motoneuron expression of NFM, Tα1-tubulin and GAP-43

The present study demonstrates that 1 hr 20 Hz electrical stimulation of the proximal stump of the axotomized and sutured femoral nerve, dramatically decreased and increased motoneuron expression of NFM and RAG (Tα1-tubulin and GAP-43) mRNA, respectively. This occurred in association with the dramatic acceleration of motor axonal regeneration and upregulation of BDNF and trkB demonstrated previously (Al-Majed et al., 2000a,b) (Chapters 2 and 3). The dramatic and rapid reduction in NFM/ Tα1-tubulin expression ratio is consistent with the proposal that "reduction in neurofilament/tubulin synthesis ratio leads to rapid induction of axonal elongation" (Hoffman and Lasek, 1980; Hoffman et al., 1985). Moreover, electrical stimulation dramatically decreased the NFM/Tα1-tubulin expression ratio during the first 2 days of regeneration, consistent with recent observation which show that there is an acceleration in elongation rate over the first

3 days following axotomy (Sjöberg and Kanje, 1990), the time when the greatest changes are occurring in the NFM/ Tα1-tubulin expression ratio.

Low frequency electrical stimulation applied immediately after surgical suture of the cut femoral nerve dramatically accelerates speed and accuracy of axonal regeneration (Al-Majed et al., 2000a) (Chapter 2). The rapid reduction of NFM and induction of RAGs expression in femoral motoneurons 2 d after 1 hr stimulation of the cut and surgically sutured femoral nerves (Fig. 4.4 and 4.7) can account for the dramatic enhancement of motor axonal regeneration. Normally 200 motoneurons regenerate their axons 25 mm from the suture site by 2 to 3 weeks. This number progressively doubled by 8 to 10 weeks (Fig. 4.3 in Al-Majed et al., 2000a) (Chapter 2). Axonal outgrowth rates correlate with the slow component b (SCb) velocity of axonal transport of tubulin. At this rate (3 mm/day), all motoneurons which are expected to regenerate their axons over 25 mm (to the point of application of retrograde dyes) in 2 to 3 weeks did not do so until 8 to 10 weeks had elapsed. This long period of 8 to 10 weeks is consistent with the delay in upregulation of Tα1-tubulin by regenerating motoneurons and the fact that the changes in gene expression of NFM and Tα1-tubulin in the cell body would take many days to affect the NFM and Tα1-tubulin content of distal axons. However the magnitude as well as time course of the reduction in NFM and the induction of Tα1-tubulin by 1 hr stimulation in association with the acceleration of axonal regeneration provides strong support for a direct link between NFM and Tα1-tubulin expression in motoneurons and axonal outgrowth. We have recently reported that 1 hr electrical stimulation promotes the speed of motor axonal regeneration. In the present study we also, show that electrical stimulation not only downregulates NFM mRNA but also greatly upregulates Tα1-tubulin and GAP-43 mRNA after femoral nerve transection and suture. This effect on gene expression of both NFM and Tα1-tubulin mRNA lead to a dramatic reduction of NFM/Tα1-tubulin expression ratio. NFM/Tα1-tubulin expression ratio in intact motoneurons is ~ 2.9 to 1. As we mentioned in the previous section, this ratio decreased to ~ 1.6 to 1 by 2 d after nerve suture and sham stimulation. In contrast, when motoneurons were subjected to 1 hr electrical stimulation this ratio dramatically declined to ~1 to11.3 by 2 d after nerve suture. This is ~ 33 times lower than intact motoneurons and ~ 18 times lower than that shown in regenerating motoneurons 2 d after suture with sham-stimulation. This dramatic reduction in NFM/Tα1-tubulin expression ratio is much greater than that induced by a conditioning lesion (Tetzlaff et al., 1996).

Axons regenerate more rapidly after a "test" lesion of a peripheral nerve if they received an earlier "conditioning" lesion (McQuarrie, 1985). This report suggests that the cell body reaction to injury is responsible for this conditioning lesion effect. Effect of electrical stimulation on tubulin gene expression differ from that of conditioning lesion. Although both accelerates axonal regeneration, only electrical stimulation upregulates tubulin mRNA after femoral nerve cut and suture. Tetzlaff et al. (1996) showed that conditioning lesion has only effect on neurofilament gene expression, but not for other cytoskeletal proteins. In particular, tubulin where changes in its synthesis are regeneration-dependent. On the other hand application of only 1 hr electrical stimulation not only downregulates neurofilament mRNA but also greatly upregulated tubulin mRNA in regenerating motoneurons. Thus 1 hr electrical stimulation reduces neurofilament/tubulin expression ratio much more than that of conditioning lesion.

In addition, electrical stimulation might increase the delivery of Tα1-tubulin to the end of the axon. McQuarrie and Grafsten (1982) have found that a conditioning lesion produced a doubling of regeneration in goldfish retinal ganglion cells. This effect is associated with increases in both protein synthesis in the neurons, and the velocity and amount of slow axonal transport. Our findings clearly support our hypothesis that the induction of speed of motor axonal regeneration by stimulation is at least in part due to the rapid and dramatic reduction in NFM/Tα1-tubulin expression ratio. Electrical stimulation also greatly upregulates GAP-43 in regenerating motoneurons 2 d after nerve suture. These findings support the "GAP hypothesis" (for review, see Skene, 1989).

The mechanism of regulation of NFM,Tα1-tubulin and GAP-43 gene expression by electrical stimulation is unknown. One possibility is that electrical stimulation regulates gene expression of these proteins indirectly via BDNF effect. This hypothesis is attractive in the context of our recent findings that stimulation rapidly and dramatically upregulates BDNF and its high affinity receptor trkB in regenerating motoneurons 8 h and 2 d after nerve suture (Al-Majed et al, 2000b) (Chapter 3). BDNF has been reported to stimulate Tα1-tubulin and GAP-43 expression in axotomized facial motoneurons that express trkB (Fernandes et al., 1995). A more recent study has reported that infusion of recombinant human BDNF into the vicinity of axotomized rubrospinal neurons (RSNs), increases the number of axotomized RSNs that regenerated into a peripheral nerve graft concurrent with upregulation of regeneration-associated genes Tα1-tubulin and GAP-43 (Kobayashi et al., 1997). Moreover, BDNF increases the intraretinal branch length of injured retinal ganglion cells of adult rats concurrent with increase mRNA level for GAP-43 (Fournier et al., 1997). The fact that upregulation of BDNF occurs at 8 hr, earlier than NFM, Tα1-tubulin and GAP-43 (2 d after nerve suture) supports our hypothesis. However more work should be done in order to elucidate the exact mechanism(s) by which electrical stimulation regulates NFM, Tα1-tubulin and GAP-43 gene expression.

4.5.4.Conclusion

We show here that the reduction of NFM and induction of Tα1-tubulin and GAP-43 gene expression by application of 1 hr electrical stimulation directly correlates with an increased number of regenerating femoral motoneurons. It remains to be elucidated whether the reduction of NFM and induction of Tα1-tubulin and GAP-43 gene expression is sufficient to induce a positive regenerative response, or whether changes in gene expression of other growth-associated proteins, e.g., CAP-23 (Widmer and Caroni, 1990) or microtubule-associated proteins (Fawcett et al., 1994; Nothias et al., 1995), might play a cooperative role in this process (Caroni et al., 1995). Irrespective, the present study indicates that the application of only 1 h electrical stimulation to axotomized and sutured femoral motoneurons decreases its NFM and increases Tα1-tubulin and GAP-43 gene expression along with enhancement of its propensity to regenerate.

4.6 REFERENCES

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CHAPTER 5

5.0 GENERAL DISCUSSION, SUMMARY AND CONCLUSIONS

5.1 SUMMARY

In this study we have used an adult rat peripheral nerve (femoral nerve) transection and surgical repair model to answer the following questions: (1) How does a transection injury of a peripheral nerve affect (a) speed and (b) accuracy of motor axonal regeneration after surgical reunion of the nerves? (2) Does low frequency electrical stimulation promote the speed and accuracy of motor axonal regeneration? (3) What is (are) mechanism(s) of action of electrical stimulation? Our results demonstrate the following: (1) (a) regenerating axons continue to reinnervate the distal pathway for protracted periods of up to 10 weeks after femoral nerve cut and surgical suture, (b) this process of gradual or staggered reinnervation is associated with progressive reinnervation of appropriate muscle pathways by regenerating motor axons (PMR). (2) We have also demonstrated that both the staggered axonal regeneration and PMR can be accelerated by electrical stimulation of the axotomized motoneurons and requires as little as 1 hr of electrical activity. (3) (a) the positive effect of 1 hr electrical stimulation is mediated at the cell body, (b) semi-quantitative *in situ* hybridization results demonstrate that short-term (1 hr) continuous 20 Hz stimulation greatly accelerates expression of the genes for BDNF and its high-affinity trkB receptor in regenerating motoneurons. The induction of BDNF gene expression by electrical stimulation is followed by concurrent reduction in mRNA level for NFM and induction of expression of the genes for Tα1-tubulin and the growth associated protein, GAP-43 in regenerating motoneurons.

5.2 SPEED AND ACCURACY OF MOTOR AXONAL REGENERATION

To avoid ambiguity and to model the most severe clinical scenario, we studied adult rats with complete surgical transection of the femoral nerve to investigate the effect of transection of peripheral nerve in the swiftness and exactness of motor axonal regeneration. We have demonstrated that motor axons slowly and progressively regenerate into the distal stumps after femoral nerve transection and surgical repair. We find that, at the initial stage of regeneration (2-3 weeks) ~ 50 % of motoneurons regenerate their axons into the distal stumps. The number of motoneurons which regenerate their axons increased progressively with time (from 4 to 10 weeks) and reached maximum at 8 to 10 weeks. This phenomenon of slow progressive axonal regeneration as staggered regeneration (Al-Majed et al, 2000a) (Chapter 2). We also find that at 2 and 3 weeks after nerve suture, regeneration of motor axons into muscle and cutaneous pathways is random. In other words, an equal number of motoneurons regenerate their axons into the appropriate muscle pathway and into the inappropriate cutaneous pathway. However, motoneurons which regenerate axons after 3 weeks regenerate into the appropriate muscle pathway. This process of precise motor axonal regeneration is called preferential motor reinnervation (PMR).

5.3 ELECTRICAL STIMULATION ACCELERATES THE SPEED AND ACCURACY OF MOTOR AXONAL REGENERATION

Electrical stimulation for as brief as 1 hr, applied immediately after surgical suture of the transected femoral nerve, markedly promoted the speed of motor axonal regeneration. One hr electrical stimulation promoted the regeneration of all motor axons over a 25 mm distance from the surgical site to maximum levels by 3 weeks as opposed to the 8-10 weeks required without stimulation. Electrical stimulation also accelerated the preciseness of motor axonal regeneration (development of PMR). The difference between the number of motoneurons projecting to muscle and cutaneous branches of the femoral nerve normally seen 8 to 10 weeks after nerve repair, was present after only 3 weeks with electrical stimulation.

5.4 MECHANISM (S) OF ACTIONS OF ELECTRICAL STIMULATION

An understanding of the mechanism(s) by which electrical stimulation promotes axonal regeneration will provide important information about the critical factors which regulate peripheral nerve regeneration. The following are the steps which we undertook to dissect the mechanism(s) of action of brief electrical stimulation:

5.4.1 Site of action

The first step in dissecting the mechanism of action by which 1 hr electrical stimulation mediates its positive effects in speed and accuracy of motor axonal regeneration is to localize its site of action. Our tetrodotoxin results indicate that the cell body is the site of action of 1 hr electrical stimulation (For more details, please see Chapter 2 or Al-Majed et al., 2000a).

5.4.2 Cell body response

Once we localized the site of action of 1 hr electrical stimulation to the cell body, we turned our focus to examine the nature of the molecular changes in the cell body in response to 1 hr electrical stimulation. We were particularly interested in whether electrical stimulation accelerates and promotes gene expression of BDNF and its high affinity receptor trkB in light of the proposed role of BDNF in supporting motoneuronal survival and regeneration along with the rapid and subtle activity-dependent upregulation of BDNF mRNA in central neurons (For more information about the rationale for using BDNF, see Chapter 3). We have found that 1 hr electrical stimulation applied immediately after femoral nerve cut and surgical repair greatly accelerates gene expression of BDNF and its high affinity receptor trkB in regenerating motoneurons. This induction of BDNF and trkB is followed by concurrent dramatic reduction in the gene expression of neurofilament and induction of Tα1-tubulin as well as GAP-43 mRNA levels. The fact that acceleration of gene expression of BDNF occurs earlier and the fact that BDNF has been shown to stimulate gene expression of Tα1-tubulin and GAP-43 (Fernandes et al., 1995; Fournier et al., 1997; Kobayashi et al., 1997) support the hypothesis that electrical stimulation modulates gene expression of these proteins indirectly via BDNF. In other words, 1 hr electrical stimulation promotes earlier upregulation of BDNF mRNA which then stimulates the reduction of gene expression of neurofilament, concurrent with induction of Tα1-tubulin as well as GAP-43 gene expression.

As we described in Chapter 4 (Al-Majed et al., 2000c), the concurrent reduction of NFM and induction of tubulin might lead to more tubulin being transported to axons which in turn leads to rapid axonal outgrowth and regeneration. We also described that the upregulation of GAP-43 after femoral nerve cut and suture is consistent with the "GAP hypothesis" (for review, see Skene, 1989), which predicts that the expression of growth-associated proteins, such as GAP-43, is a prerequisite to successful axonal regeneration. Given the importance of these proteins in axonal regeneration, it will be useful to further characterize the mechanism by which BDNF might regulate their expression. The signalling pathways that mediate BDNF regulation of NFM and RAGs (Tα1-tubulin and GAP-43) gene expression, which may in turn accelerate the number of axons which regenerate, are unknown. Several downstream cascading pathways have been defined that play major roles in BDNF signalling (for review, see Green and Kaplan; 1995; Segal and Greenberg, 1996; Kaplan, 1998; Friedman and Greene, 1999; Klesse and parade, 1999). Foremost among these is the Ras/raf/MEK/ERK pathway which leads to increased gene transcription. It also plays a major role in mediating neurite outgrowth and other responses to BDNF. One possible mechanism by which BDNF stimulates gene expression of Tα1-tubulin and GAP-43 is by stimulating the ras pathway. Further experimentation will be required to determine which pathway (s) is (are) critical for BDNF regulation of RAGs expression.

5.4.3 Electrical stimulation, specificity and L2/HNK-1

A possible mechanism for the promotion of PMR by electrical stimulation is by increasing the number of motoneurons which successfully regenerate their axons across the surgical gap into the endoneurial tubes of the distal nerve stump. This is possibly achieved by the reduction of motoneuron NFM concurrent with the induction of Tα1-tubulin. Thus, more motor axons would be available to respond to specific cues which have been identified in the muscle pathways (e.g. L2/HNK1) and thereby accelerate the emergence of preferential reinnervation of the muscle pathway. Support for this hypothesis is provided by our recent findings in mice which demonstrate that 1 hr electrical stimulation elevates the L2/carbohydrate expression in the Schwann cells of only the muscle (motor) pathways within a week of nerve transection and suture (Al-Majed et al., 2000d). Normally L2 expression is significantly reduced in the Schwann cells of the motor pathways. Conversely, electrical stimulation accelerated the downregulation of L2 in Schwann cells of the cutaneous pathways. These findings suggest that accelerated PMR by electrical stimulation is due, at least in part, to more rapid L2 upregulation in motor Schwann cells and in turn, to specific attraction of motor axons into muscle pathways by these cells (Al-Majed et al., 2000d).

5.4.4 Sensory input

Although our data strongly suggest that the positive effects of 1 hr electrical stimulation are directly mediated via the motoneuronal cell body (Al-Majed et al., 2000a-c or see Chapter 2-4), we cannot exclude the possibility that this effect is mediated indirectly by electrical stimulation of sensory neurons in dorsal root ganglia which in turn, send anterograde positive signal(s) to motoneurons leading to upregulation of BDNF mRNA. This possibility is supported by increasing evidence that BDNF is anterogradely transported by central and peripheral neurons, in response to activity (Heymach et al., 1996; Altar and DiStefano, 1998; von Bartheld, 1998; Griesbeck et al., 1999; for review, see Goldberg and Barres, 2000). In addition, axotomy increases the synthesis and anterograde transport of BDNF from sensory neurons (Tonra et al., 1998; for review, see Terenghi, 1999) and it has been postulated that it might act as an anterograde trophic messenger by being released in the dorsal horn under the influence of NGF and modulating nociceptive signaling (Michael et al., 1997). In addition, a recent study has indicated that electrical stimulation of dorsal roots resulted in changes in the polysynaptic potentials recorded from the motoneurons (See Bach and Mendell, 1996). Based on these findings, a hypothetical mechanism by which 1 hr electrical stimulation mediates its positive effects is by electrically stimulating dorsal roots which, in turn, anterogradely stimulate the production of polysynaptic potentials in motoneurons. As discussed in Chapter 3, brief depolarization induces BDNF gene expression (for more details about depolarization and upregulation of BDNF mRNA, please see Chapter 3 or Al-Majed et al., 2000b). Thus, the production of polysynaptic potentials might lead to induction of BDNF gene expression in motoneurons. The most direct approach to investigate this possibility would be by depriving the femoral motoneuron of sensory innervation by deafferentation (excision of dorsal root ganglia of lumbar segments 2-4) (Martini et al., 1994) followed by application of 1 hr electrical stimulation, evaluation of speed and accuracy of motor axonal regeneration and measurement of motoneuronal BDNF gene expression.

5.5 FUTURE DIRECTIONS

The use of 1 hr electrical stimulation in promoting peripheral nerve regeneration after nerve cut and surgical repair has a strong potential for clinical use. However, further understanding of the mechanism(s) of action of 1 hr electrical stimulation on motor axonal regeneration is required before we may extend our experimental findings to practical usage. I propose that this further understanding may be achieved by the following:

5.5.1 Examining the direct role of BDNF in the positive regenerative response of 1 hr electrical stimulation

Although we have reported an increase in BDNF mRNA level after 1 hr electrical stimulation and the fact that there is a very good agreement between mRNA (using *in situ* hybridization) and protein levels (using immunocytohistochemistry) of BDNF (Kobayshi et al. 1996), we need to provide more direct evidence of the role of BDNF in the positive regenerative response of brief electrical stimulation. One way of investigating the direct role of BDNF in the positive effects of electrical stimulation is by suppressing BDNF upregulation by gene deletion, introduction of BDNF antisense oligoncleotides or treatment with BDNF antibodies. These tools have been proven to be a reliable way to test the involvement of BDNF in several neuronal functions. For example, introduction of BDNF antisense oligoncleotides resulted in deficiencies in hippocampal long-term potentiation (LTP); this can be reduced by acute provision of exogenous BDNF (Korte et al., 1995,1996a,b; Patterson et al., 1996). In addition, intracerebroventicular infusion of antisense oligoncleotides sequence to BDNF blocked the upregulation of BDNF induced by kainic acid and significantly increased the seizure duration induced by kainic acid (Tandon et al., 1995). In addition, survival of cultured dorsal root ganglion cells (Acheson et al., 1995) and cortical neurons (Ghosh et al., 1994) were significantly decreased by treatment with BDNF antisense oligoncleotides and with BDNF antibodies, respectively.

5.5.2 Usage of pharmacological agents which augment BDNF synthesis by motoneurons

A pharmacological augmentation of BDNF synthesis would represent an attractive alternative to the invasive administration of BDNF (for review, see Thoenen et al., 1994). Several pharmacological agents have been shown to increase BDNF mRNA level. For example, intraperitoneal administration of kainic acid, an agonist of the non NMDA-type glutamate receptors increased BDNF mRNA

(Zafra et al., 1990; Dugich-Djordjevic et al., 1992). This increase in BDNF mRNA was apparent as early as 1 hr after kainic acid administration (Zafra et al., 1990). Bicuculline, an antagonist of $GABA_A$ receptors strongly increased BDNF mRNA level (Zafra et al., 1991). Furthermore, agonists of muscarinic receptors increase BDNF mRNA level. For example, administration of the muscarinic agonist pilocarpine to rats produced a robust increase in BDNF mRNA (Berzaghi et al., 1993). Histamine was also found to increase BDNF mRNA level (Zafra et al., 1990). In addition, agents which increase intracellular cyclic AMP levels, such as forskolin or norepinephrine have been shown to stimulate BDNF expression (Zafra rt al., 1992). Furthermore, Ca^{2+} ionophores and activators of L-type calcium channels increase BDNF gene expression (Zafra et al., 1992). Administration of more than one agent has been shown to produce high level of BDNF mRNA. For instance, glutamate, which on its own has no effect on BDNF mRNA level, potentiates the forskolin-mediated increase in BDNF mRNA (Zafra et al., 1992). Furthermore, when forskolin was given together with kainic acid or calcium ionophores, it markedly enhanced the effects of the latter substances (Zafra et al., 1992).

5.5.3 Measurement of rate of slow component b after 1 hr electrical stimulation

To further support the involvement of the major cytoskeletal protein, Tα1-tubulin in the positive effect of electrical stimulation on axonal regeneration, I propose to test the hypothesis that 1 hr electrical stimulation promotes axonal regeneration by increasing the delivery of tubulin via an increase in the slow component b (SCb) rate to the axon tip. The SCb is the most rapid vehicle for carrying cytoskeletal proteins to the axon tip and an increase in this rate would produce a corresponding change in the axonal outgrowth rate. To test this hypothesis, the most direct approach is to apply 1 hr electrical stimulation and measure the SCb rate of axonal transport. During regeneration in the adult, there is a correlation between SCb velocity containing tubulin and axonal outgrowth rate (Wujek et. al., 1983). Upregulation of tubulin concurrent with rapid downregulation of neurofilament expression (mRNA and protein levels) has been suggested to decrease the neurofilament/tubulin synthesis ratios, allowing more rapid transport of tubulin and more rapid axonal elongation (Hoffman and Lasek, 1980; Hoffman et al., 1985). It has been proposed that axonal elongation relies upon the supply of tubulin delivered in SCb to the growth cone (Jacob and McQuarrie, 1991). In fact, the increased axonal regeneration rate induced by conditioning lesion was accompanied by increased delivery of tubulin to the end of axons by SCb (McQuarrie and Grafstein, 1982; McQuarrie and Jacob, 1991). Thus an increase in the velocity of tubulin in SCb would support the faster axonal regeneration of electrically stimulated axons.

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